

LOCALISATION OF TISSUE ANTIGENS WITH THE
FLUORESCENT ANTIBODY TECHNIQUE: STUDIES
ON CONNECTIVE TISSUE, THE KIDNEY AND
ANTERIOR PITUITARY HORMONES.

By

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Thesis presented for the Degree of Doctor
of Medicine, September, 1958.



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SECTION I.

INTRODUCTION

INTRODUCTION

Chemical labelling of antibodies, to enable the site of antigen-antibody reactions to be visualised, was introduced by Marrack (1934) who used a visible dye attached to the antibody by an azo linkage. Two other types of label have been introduced, namely, fluorochromes and radio-isotopes. Coons et al (1941) used an anthracene derivative conjugated to antipneumococcal serum, but found that the blue colour emitted after excitation by ultra-violet light was distinguishable only with difficulty from the natural blue autofluorescence of most animal tissues (Hamperl, 1934; Sjöstrand, 1945-6). They found subsequently (Coons et al., 1942) that fluorescein isocyanate could be conjugated to antibody protein and that its brilliant apple-green fluorescence was readily identifiable in the tissues. This method - the fluorescent antibody technique - permits very accurate histological localisation by the naked eye but cannot be used for quantitative studies. Essentially it involves performing a precipitin test on tissue sections with the fluorochrome-labelled antibody and examining the sections in the fluorescence microscope. The labelling of antibodies/

antibodies with radio-isotopes, introduced by Pressman & Keighley (1948), has the advantage of greater sensitivity than either of the other types of label, thus enabling accurate quantitative work. However, the tissues cannot be examined with the naked eye and the definition of autoradiographs is as yet inferior to that obtainable with antibodies labelled with fluorescein. The use of labelled antibodies to detect sites of antigen-antibody reactions in the tissues is a form of histochemistry - immunohistochemistry - which is being applied to a rapidly expanding field. It provides a method by which proteins and other antigens, distinguishable with difficulty, or not at all, by other methods, can be specifically differentiated from each other. The main applications of the fluorescent antibody technique have been the fate of foreign antigens injected into experimental animals, the identification of viruses and bacterial products in the tissues, the identification of sites of antibody formation and the localisation of normal tissue components.

The main purpose of this thesis is to describe experiments in the last-named group, in particular the localisation of connective tissue and kidney components in man. There have been remarkably few/

few immunological studies of normal human tissues, so it was necessary to apply the method to such tissues before using it for studies on pathological material. The two largest sections of the thesis are derived from a study of antigenic components of rat kidney (Hill & Cruickshank, 1953): Section II describes studies on several normal connective tissue components, namely basement membrane, reticulin, collagen and chondroitin sulphate, and Section III describes studies of the antigenicity of normal human and monkey kidney and the application of the fluorescent antibody technique and other immunological methods to an investigation of acute glomerulonephritis and anaphylactoid purpura for auto-antibodies to normal kidney and blood vessel components. Section IV - an attempt to produce specific antibodies to several human anterior pituitary hormones - is not connected with the rest of the work other than by the methods used. It is included because it illustrates some of the difficulties involved in immunological and immunohistochemical studies of tissue antigens. The thesis ends with a general discussion of the work and suggestions for further work (Section V).

The localisation of a tissue component with/

with the fluorescent antibody technique entails a complicated series of procedures, namely:

- a) preparation of a tissue, or extract therefrom, for
- b) immunisation of animals;
- c) analysis of the immune serum to identify antibodies and remove those not required;
- d) preparation and attachment of the fluorescent label;
- e) preparation of tissue sections for
- f) treatment with the labelled antiserum, or its globulin fraction;
- g) establishment of the specificity of the reaction in the tissues;
- h) examination of sections in the fluorescence microscope;
- i) identification of the site of staining^{*} with histological or histochemical methods.

The technical procedures and animal protocols for the present work will be described separately from the main text, in the Appendix. The procedure for establishment of specificity of staining will be found on pp.8 - 11.

* "Staining" is not a strictly accurate term for a procedure which results in the appearance in sections of an antigen-antibody precipitate identifiable in ultra-violet light by virtue of a fluorochrome attached to the antibody. However, it is a convenient and short way of describing both the procedure and the presence of specific fluorescence in treated tissues and is accepted in the literature. It will be used throughout this thesis in this as well as its more usual sense.

SECTION II.

IMMUNOLOGICAL AND IMMUNOHISTOCHEMICAL

STUDIES ON CONNECTIVE TISSUES

BASEMENT MEMBRANE AND RETICULIN

Since the experiments of Pearce (1903-04) evidence has been gradually accumulating that renal cortex contains an antigenic component capable of provoking, in another species, antibodies which will react with vascular tissue in the original species. During the last decade attempts have been made to identify the tissue component which is responsible. Heymann & Lund (1948) confirmed Pearce's observation that the cortex contains more of this component than the medulla, and Solomon et al. (1949) discovered that isolated glomeruli could effectively absorb the antibody from anti-kidney serum. The antibody content of such a serum can be demonstrated in vivo by its nephrotoxicity, that is the production of a form of nephritis which in many ways resembles human glomerulonephritis. Greenspon & Krakower (1950) discovered that isolated glomeruli were able to produce a potent nephrotoxic serum, whereas other components of the renal cortex were ineffective. These workers (Krakower & Greenspon, 1951) subsequently immunised animals with various components of glomeruli, separated by ultrasonic vibration, and concluded that the/

the basement membrane of the tufts accounted for most of the antigenic activity of whole glomeruli. Further information on the source of the antigen was obtained by Pressman et al. (1949), who injected anti-mouse-kidney globulin labelled with ^{131}I into living mice and found a high concentration of radioactivity in the glomeruli.

There is evidence that one, or more, of the antigens in the glomerulus is present in other tissues. Thus anti-lung (Chikamitsu, 1940), anti-placenta (Seegal & Loeb, 1946) and anti-aorta (Strehler, 1951) sera have proved to be nephrotoxic, and Pressman and his colleagues have shown that ^{131}I -labelled antisera to rat lung (Pressman & Eisen, 1950a), rat liver blood vessels (Pressman et al., 1951) and rat aorta (Pressman et al., 1952) all localise in kidney, lung and liver.

Hill & Cruickshank (1953) applied the fluorescent antibody method to this problem and were able to demonstrate two separate antigens in the rat kidney - one in glomerular basement membrane, the other in the epithelium of the convoluted tubules - and that anti-rat-lung serum contained an antibody to the basement membrane/

membrane antigen. This section of the thesis describes the extension of that work to studies on basement membrane and reticulin in many tissues of the rat and, later, man.

BASEMENT MEMBRANE AND RETICULIN IN THE RAT

METHODS

Antisera were prepared in rabbits by immunisation with suspensions of perfused whole rat kidney (Rabbits 42-44), isolated rat glomeruli (Rabbit 47), perfused rat lung (Rabbit 46), and alum-precipitated rat serum (Rabbit 49). The details of preparation of the antigens, immunisation of the rabbits and titration of the antisera are described in the Appendix (pp. 186, 191-2, 198). Immunisation with the organ extracts was continued until the antisera gave a strong precipitin reaction with a saline extract of the relevant organ. Cross-reactions between the anti-organ sera and rat serum and erythrocytes were eliminated by appropriate absorptions (see Appendix, p. 199). Sera, or their globulin fractions, were conjugated with fluorescein isocyanate by the method of Coons & Kaplan (1950) (see Appendix, p. 201). Fresh-frozen tissues were/

were used throughout the work and were obtained from adult rats killed by ether. The method of preparing, treating and examination of the tissues was essentially similar to that used by Coons and his collaborators (see Appendix, p. 202).

Specific staining due to fixation of fluorescent antibody by its homologous tissue antigen was seen as bright green fluorescence. Tissues in which no reaction had occurred were visible because of their autofluorescence, which varied from dull blue in most tissues to bright bluish-white in elastic tissue. Ceroid, particularly in the liver and kidney, could be identified by its yellow autofluorescence.

The specificity of the green fluorescence was tested in several ways (Table I). It was necessary to establish that the staining was dependent upon a factor (antibody) present in the immune sera, but not in normal rabbit serum, and that this factor was active in the absence of the fluorescein label. This was accomplished by an inhibition test. Sections were exposed to unlabelled antiserum and to normal rabbit serum respectively for 3 hours, and thereafter treatment of both sections for 1-1½ hours with fluorescent globulin prepared from an antiserum. Fluorescent/

TABLE I

Specificity of staining with anti-glomerulus globulin

Labelled Antiserum	Staining reaction in rat glomeruli
Anti-rat-glomerulus globulin (AG)	+
Anti-rat-lung globulin (AL)	+
AG or AL after pre-treatment with unlabelled AG or AL	-
AG or AL after pre-treatment with unlabelled normal rabbit globulin	+
Normal rabbit globulin	-
AG absorbed with rat kidney	-
AG absorbed with rat lung	-
AG absorbed with rat spleen	-
AG absorbed with pig kidney	+

Fluorescent staining failed to develop in the first of these sections, indicating that the reactive groups of the antigen had been specifically bound by the unlabelled antibody, but appeared as usual in the second. As a further check on the absence of the specific antibody from the serum of a non-immunised rabbit, a third section was treated with a conjugate prepared from such a serum and was found to show no staining.

Absorption tests were also used to prove the specificity of the staining. Thus 3 parts of labelled anti-rat-glomerulus serum and 1 part of a wet, packed suspension of rat kidney were mixed and allowed to stand for 1 hour at room temperature. The preparation was spun and the process repeated. No staining occurred when this absorbed, labelled serum was applied to sections. Similar results were obtained when the serum was absorbed with rat lung or rat spleen. Absorption with heterologous (pig) kidney had no effect upon staining.

The full battery of tests for specificity was carried out only with sections of kidney, skin, trachea, thyroid and lung. With other tissues, staining was regarded as specific if it appeared only in the first and last of four sections treated with:-

- a) fluorescent immune rabbit globulin,
- b) fluorescent normal (non-immune) rabbit globulin,
- c) unlabelled immune globulin, followed by fluorescent immune globulin (specific inhibition), and
- d) unlabelled normal rabbit globulin followed by fluorescent immune globulin.

The species specificity of the reaction was investigated by applying fluorescent anti-rat-kidney serum to sections of mouse, guinea-pig, rabbit, monkey and human kidney. Staining was produced in the mouse similar to that in the rat, whereas no reaction occurred in the other species. An immunological cross-reaction between rat and mouse kidney has been noted previously (Pressman, 1949).

The following rat tissues were treated with anti-rat-kidney (AK), anti-rat-glomerulus (AG) or anti-rat-lung (AL) globulins:- heart (AK), femoral artery (AK, AG), trachea (AK, AG, AL), lung (AK, AG, AL), tongue (AK, AG), submandibular gland (AK), oesophagus (AK), stomach (AK), small intestine (AK), colon (AK), liver (AK), pancreas (AK), kidney (AK, AG, AL), bladder (AK), testis (AK), ovary (AK), uterus (AK), uterine tube (AK), placenta (AK), breast (AK, AL), spleen (AK, AL), lymph node (AK, AG, AL), thymus (AK), pituitary (AK), adrenal (AK, AG), thyroid (AK, AG, AL), parathyroid/

parathyroid (AG), brain (AK,AG), peripheral nerve (AK,AG), skin (AK,AG,AL), skeletal muscle (AK,AG), tendon (AK,AG), lacrimal gland (AK). Many of these tissues were treated with two separate antisera to rat kidney, with identical results.

The pattern of staining obtained with the fluorescent sera was compared with that of adjacent sections stained with haematoxylin and eosin or the periodic acid-Schiff technique (McManus, 1946), counterstained with haematoxylin. It was sometimes impossible to obtain satisfactory results with the periodic acid-Schiff technique on fresh-frozen sections and even haematoxylin and eosin staining was difficult to interpret accurately. For this reason, identification of structures in serial sections was not always possible and some of the conclusions, particularly regarding sites of non-specific staining, are tentative. Much more satisfactory results with histological and histochemical procedures were obtained in later work (see p. 149).

RESULTS

The pattern of specific fluorescent staining obtained with the sera prepared against rat kidney, rat glomeruli and rat lung was, except/

except for the kidney, the same in all the tissues studied. In the kidney, the anti-glomerulus and anti-lung globulin produced identical staining of basement membranes and reticulin, whereas the anti-kidney globulin also stained the cytoplasm of the cortical tubular epithelium. This peculiarity of the anti-kidney globulin is not relevant to the present discussion and will not be considered further. No specific staining occurred in the cells of other tissues and staining of nuclei was not observed. These results have been described by Cruickshank & Hill (1953a).

Basement Membrane

Fluorescent staining of basement membrane occurred in the trachea (Fig.1), intra-pulmonary branches of the bronchial tree, tongue, submandibular gland (Fig.2), oesophagus, stomach, small intestine, colon (Fig.3), intrahepatic bile ducts, kidney (Figs.4-6), bladder (Fig.7), testis, ovary, Fallopian tube, uterus (Fig.8), breast (Fig.9), skin (Fig.10), thyroid (Figs.11-13) and lacrimal gland.

The membrane varied in thickness in the different organs, being thinnest beneath squamous epithelium (Fig.10) and thickest in the uterus (Fig.8). In most situations only a single/

Figs. 1 - 40 and 48 are photomicrographs of tissues treated with fluorescein-labelled immune globulins. The general structure of each tissue is seen by virtue of its dull blue auto-fluorescence. White lines and masses are due either to specific apple-green fluorescence at sites of antigen-antibody union, or to the bright bluish-white auto-fluorescence of elastic tissue.

AK - anti-kidney: AG - anti-glomeruli: AL - anti-lung.

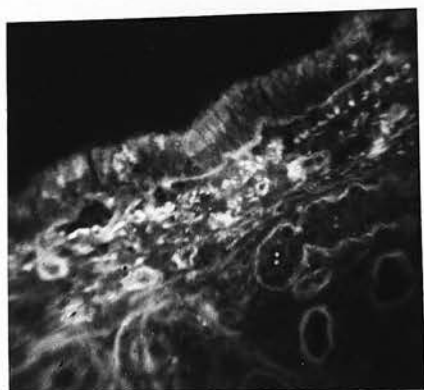


Fig.1. Rat trachea. The basement membrane is seen as an almost complete line deep to the columnar epithelium. The bright fragments beneath it are elastic tissue. In the lamina propria specific staining is seen in the basement membrane of a gland (centre right) and in the reticulin of capillaries. AK (x 300).

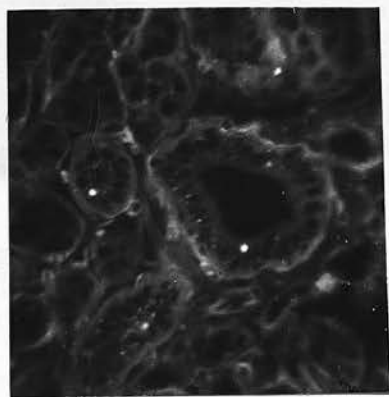


Fig.2. Rat submandibular gland. Basement membrane is seen around acini and a duct. AK (x 300).

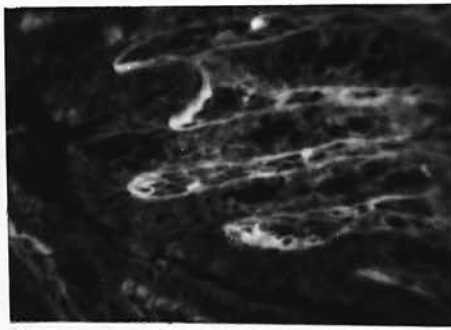


Fig.3. Rat colon. Basement membrane beneath epithelium and reticulin in capillaries of the villi. AK (x 300).

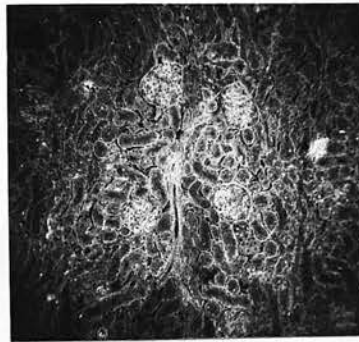


Fig.4. Rat kidney. Basement membrane of glomeruli and convoluted tubules is stained. AG (x 50).

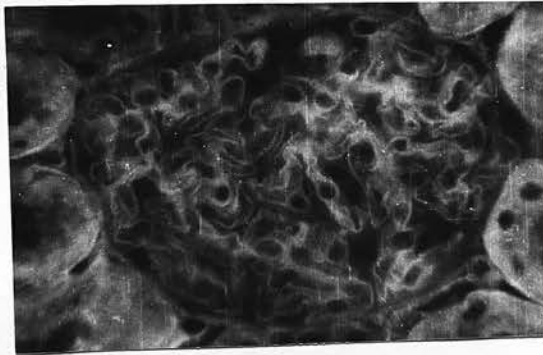


Fig.5. Rat glomerulus. The basement membrane of the tuft, of Bowman's capsule and of convoluted tubules is seen as a single line. Epithelial cells in the glomerulus and tubules are also stained specifically as the section was treated with anti-kidney globulin. (x 385).

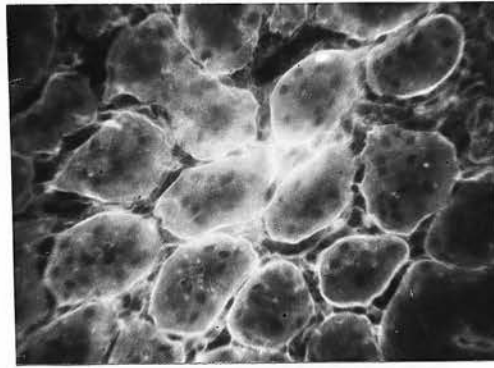


Fig.6. Rat kidney. Basement membrane of convoluted tubules and reticulin of capillaries are seen. AG (x 250).

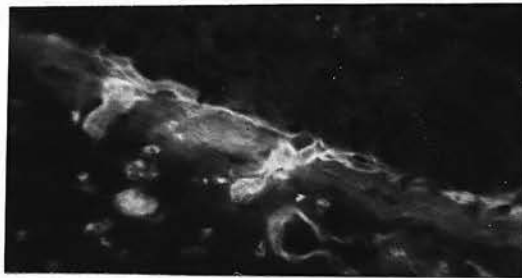


Fig.7. Rat bladder. Subepithelial basement membrane and capillary reticulin are stained. AK (x 250).

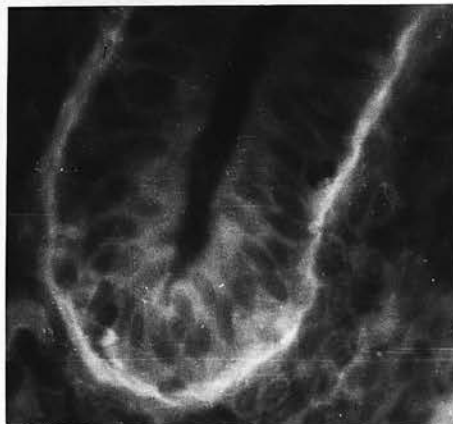


Fig.8. Rat uterus. Thick subepithelial basement membrane. AK (x 500).

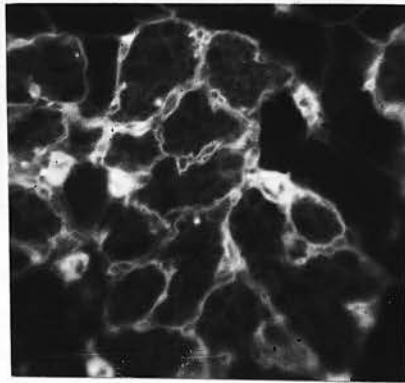


Fig.9. Rat breast, shortly before parturition. Note the splitting of the acinar basement membrane to enclose unstained nuclei. AK (x 250).

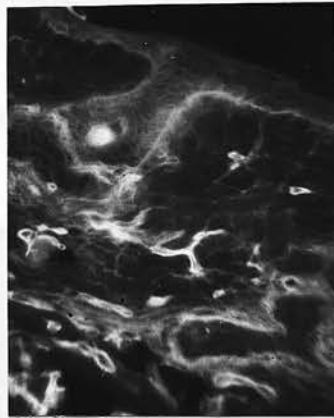


Fig.10. Rat skin. A basement membrane is seen beneath the epidermis and around a sweat gland (bottom right). Capillary reticulin stains brightly. AK (x 250).

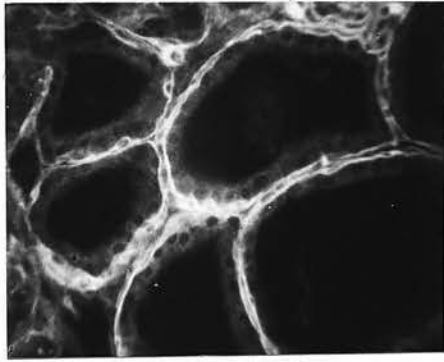


Fig.11. Rat thyroid. A complete basement membrane surrounds the acini and splits occasionally to enclose unstained nuclei. AK. (x 250).

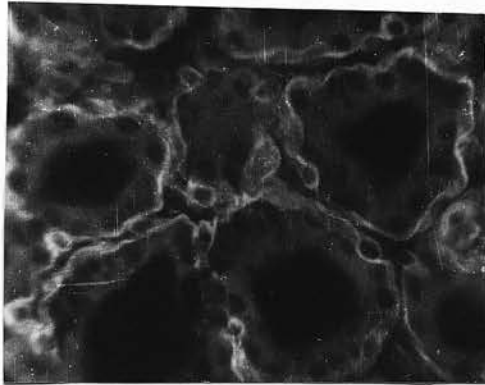


Fig.12. Rat thyroid. The splitting of the acinar basement membrane is seen even better here. AK (x 365).

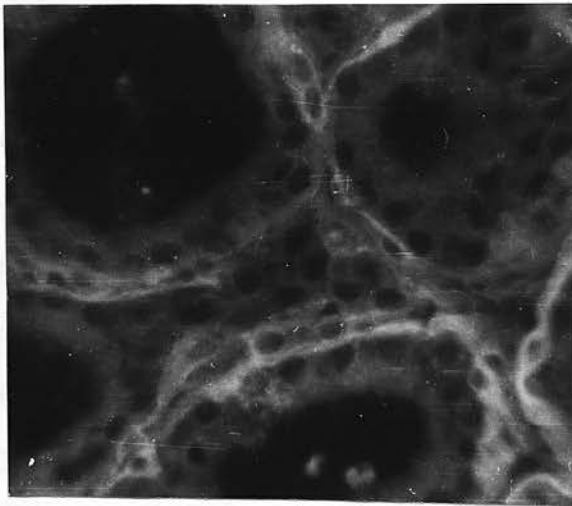


Fig.13. Rat thyroid. Appearance similar to Figs. 11 & 12, but obtained with anti-lung globulin. (x 520).

single layer could be demonstrated, but this layer occasionally split to enclose an unstained space. This feature was noted in the thyroid (Fig.12) and in the breast (Fig.9), where, in haematoxylin and eosin-stained sections, the space appeared to contain nuclei, smaller and more hyperchromatic than those of the acini, spindle-shaped or oval and arranged tangentially to the acini. In the seminiferous tubules of the testis and in the ducts of the breast, the basement membrane had two layers. In the trachea, alimentary canal, uterus and skin, a membrane was present not only beneath the lining epithelium but also around the glands and ducts in the underlying tissue (Fig.1). In exocrine glands, it was seen around both acini and ducts (Fig.2). There was considerable variation in the appearance of the glandular basement membrane in the alimentary canal. Thus, the membrane was distinct in the necks of the glands of the gastric mucosa, but could be seen only with difficulty in their deeper parts. In the small intestine the membrane was more easily seen in the crypts than in the villi; in the colon it was easily seen throughout the mucous membrane. In the bladder (Fig.7), the membrane was single in some places and double elsewhere. The basement/

basement membrane of the ovarian follicles was distinct, but none was seen in the zona pellucida of the ova. However, the integrity of the ova was poorly preserved, since, in adjacent quick-frozen, unfixed sections stained with haematoxylin and eosin, they were seen only indistinctly. In the kidney, no more than a single membrane was detected in the glomerular tufts lying between the unstained endothelium and the faintly stained epithelium (Fig.5). The basement membrane of Bowman's capsule was also stained, but was thinner than those of the tuft or the tubules. All cortical tubules had a well-defined basement membrane (Fig.6), but in the medulla this was faint and irregular so that it was impossible to identify the type of medullary tubule around which staining occurred.

Completely satisfactory sections of lung could not be obtained, partly on account of the difficulty of cutting quick-frozen unfixed sections of this tissue and also because collapse of the organ made the interpretation of the staining of fine structures difficult. So far as could be seen, no alveolar basement membrane was present and staining in the alveolar walls occurred/

occurred only in the reticulin of the capillaries (Fig.14). The basement membrane of small intrapulmonary branches of the bronchial tree was finer than in the larger extrapulmonary branches and lay close to a more or less complete lamina of elastic tissue. It was thus not always easy to differentiate these two layers.

Reticulin

A reticular network was demonstrated in the spleen (Figs.15 & 16), lymph nodes and thymus, in the walls of capillaries (Figs.1,2, 7,10,12 & 16), brain, arterioles (Fig.15) and sinusoids (Figs.17 & 18), and around plain muscle fibres in all situations where that tissue was observed (Fig.19). In the spleen (Figs.15 & 16), lymph nodes and thymus a fine network outlined the individual cells, both in the pulp and in the lymphoid follicles. Sinuses in the lymph nodes were outlined by a definite membrane, which was sometimes double. No such membrane was seen in the splenic sinuses, but the reticulum cells of both lymph node and splenic sinuses were outlined in the same way as cells in the pulp.

Capillaries in all tissues were outlined by a single fluorescent reticular membrane. The internal elastic lamina of arterioles was surrounded by one or more concentric laminae of reticulin/

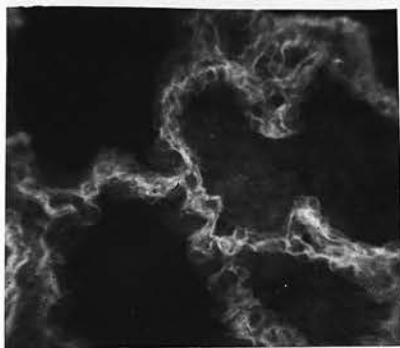


Fig.14. Rat lung. No basement membrane is seen beneath the alveolar epithelium, staining being confined to the capillary reticulin. AK (x 300).

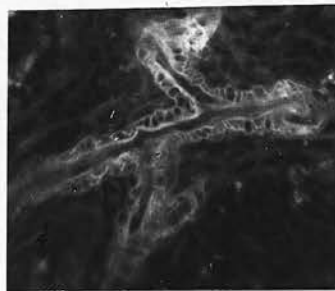


Fig.15. Rat spleen. Longitudinal section of an arteriole showing reticulin around the muscle fibres of the media, outside the continuous elastic lamina. AK (x 250).

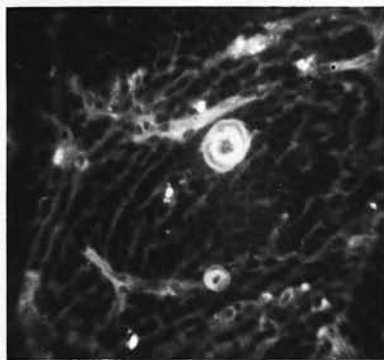


Fig.16. Rat spleen. Two arterioles in cross section in a Malpighian corpuscle with several concentric reticulin laminae around the elastic lamina. The reticulin of the corpuscle is seen as a fine pericellular network. (x 250).

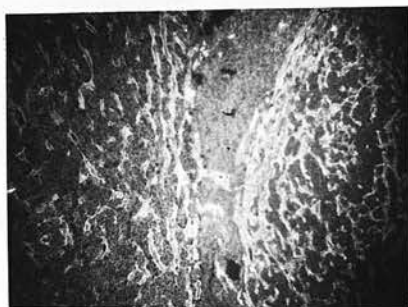


Fig.17. Rat pituitary. Bright staining of reticulin of sinusoids in pars anterior, pars intermedia and pars posterior (from left to right). AK (x 50).

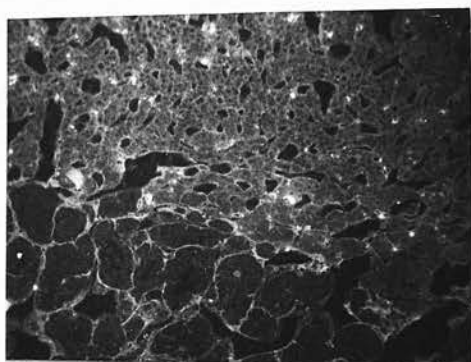


Fig.18. Rat adrenal. Rather faint reticulin in sinusoids in cortex (above) and brighter reticulin around clumps of cells and sinusoids in medulla (below). AG (x 250).

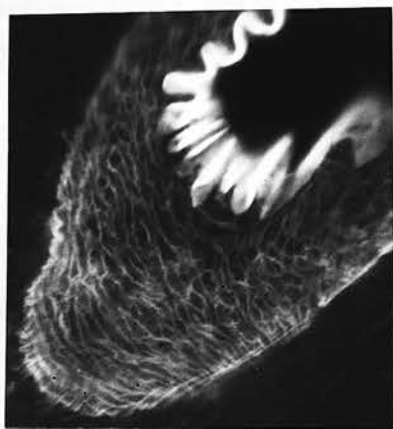


Fig.19. Rat femoral artery. The internal elastic lamina is distinctly seen as a serpiginous line, the external elastic lamina less distinctly as the outermost fluorescent layer. Between the two, the muscle fibres of the media are outlined by a brightly stained network of reticulin fibres. AK (x 265).

reticulin (Fig.16), whilst the plain muscle fibres of the media of larger vessels were outlined by a network limited by the two elastic laminae (Fig.19). Sinusoids in the pituitary (Fig.17) and adrenal (Fig.18) were limited by a distinct membrane, but, in the liver, this was clearly seen only in the neighbourhood of portal tracts or central veins. Capillaries and other vessels in several organs were separated from the parenchyma by strands of reticulum, as in the parathyroid and thymus glands. In the placenta, a very delicate reticular network surrounded capillaries in the labyrinth, whereas a much coarser membrane covered the outer surface and outlined the septa which dip into the labyrinth. No specific staining was seen in the decidua.

Plain muscle was readily recognisable by the reticular network which surrounded its fibres. This was seen in larger vessels, as noted above, and in the trachea, oesophagus, muscularis mucosae and muscular layers of the stomach and intestines, bladder and uterus and in the trabeculae of the spleen. Reticulin was also demonstrated between the cells of adipose tissue (Fig.20), in the serous coat of the alimentary canal/

canal, in the leptomeninges and in the adrenal capsule.

Other Connective-tissue Components

The sarcolemma of striated (Fig.21) and cardiac muscle was readily demonstrated and could be distinguished from reticulin in the walls of the capillaries. The pattern of staining in peripheral nerves is seen in Fig.22. The nerve bundles are surrounded by several fine concentric laminae and the individual fibres by a single layer lying outside the myelin sheath. No staining was seen in other connective-tissue elements, such as collagen fibres, elastic fibres, cartilage matrix, or ground substance, although these were encountered in many situations. Unstained collagen, which was readily differentiated from the specific green staining of basement membrane reticulin by its blue autofluorescence, was seen in the trachea, tongue, oesophagus, stomach, intestines, skin (Fig.10), ovary, uterus, capsule of spleen, adventitia of vessels (Fig.19) and tendon (Fig.21).

Miscellaneous Investigations

Accurate chemical identification of the antigen was not attempted. The absence of a reaction/

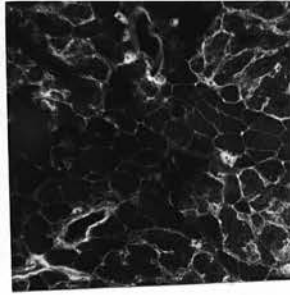


Fig.20. Rat breast. Fat cells (centre) are surrounded by reticulin fibres. (x 50).

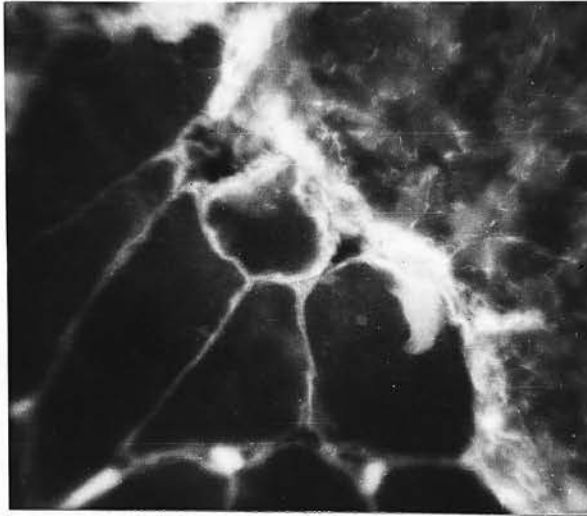


Fig.21. Rat skeletal muscle and tendon. The sarco:plasm is unstained but the sarcolemma of each fibre is clearly seen. The collagen of the tendon is unstained, but is pervaded by irregular fine strands of elastic tissue. AK (x 400).

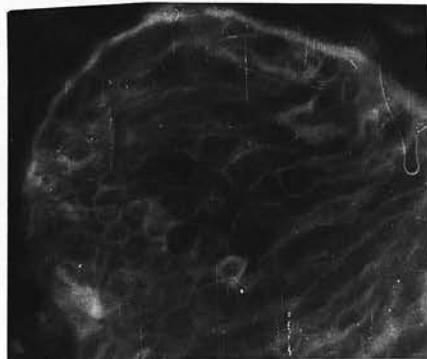


Fig.22. Rat nerve. The nerve fibres are recognisable by the faint dark blue autofluorescence of the myelin sheath, outside which is the specific staining of the neurilemma. The whole nerve is also surrounded by specifically stained fibres.

AK (x 350).

reaction in the matrix of tracheal cartilage suggested that it was not chondroitin sulphuric acid-A (Meyer, 1951). In order to determine whether hyaluronic acid was concerned, a section of rat kidney was treated with hyaluronidase (Benger's Hyalase), 1000 units per ml. of phosphate buffer, at pH 7.6 for 23 hours at room temperature before staining with fluorescent anti-rat-kidney rabbit globulin. The pattern of staining was not altered by the enzyme treatment. The activity of the enzyme preparation was confirmed by the mucin-clot-prevention test with human synovial fluid (Warren & Durso, 1951).

Non-specific Staining

The green fluorescence described so far was all due to specific precipitation of labelled antibody. Precipitated fluorescein was also seen in sites where it did not fulfil the criteria for specificity. It was seen in the cytoplasm of large "plasma cells" which were most numerous in the interstitial tissue of the tongue and were present in small numbers in the interstitial tissue of the bladder, uterus, breast, thyroid, heart, skeletal muscle, oesophagus and lung. They/

They were also seen in some lymph nodes. These cells were considerably larger than human plasma cells, measuring $120\mu \times 90\mu$, but had the same cytoplasmic and nuclear appearances in sections stained with haematoxylin and eosin as human plasma cells. Similar staining was seen in the cytoplasm of cells, which were probably eosinophils, in the mucous membrane of the stomach, small intestine and colon and in the spleen and lymph nodes. Non-specific staining of this pattern has been described by other workers since this study was completed and can be eliminated by absorbing the labelled antiserum with acetone-dried bone marrow powder (Sheldon, 1953; Coons et al., 1955). Other sites of non-specific staining encountered in the present work were the cytoplasm of tracheal and bronchial epithelium (Fig.1) and a membrane lining the lacunae of tracheal chondrocytes. No attempt has been made to study any of this non-specific staining in detail.

Discussion of these results will be deferred until similar experiments with human tissue have been described (see p. 41).

BASEMENT MEMBRANE AND RETICULIN IN MAN

The results of the study of these tissue components in the rat indicated that the fluorescent antibody method was applicable to studies of normal connective tissue antigens and that a histochemical method of immunological specificity was available. It was therefore decided to repeat the observations in human tissue for several reasons:-

- a) the method might be applicable in diseased as well as healthy tissues,
- b) the specificity and brilliance of staining suggested that information might be obtained which was not possible with other staining methods, and
- c) it was desirable to get more information about the nature of the antigen. If human postmortem tissues were antigenic, material for chemical and other analyses would be available in much greater quantity than if rats were used.

METHODS

Antisera were prepared in rabbits by immunisation with a suspension of human glomeruli isolated from kidneys obtained at autopsy (Rabbits 48 & 52) and with alum-precipitated human serum (Rabbit 51). The procedures used in preparation of antigens, immunisation of rabbits, labelling of sera, preparation, staining and examination of tissues were similar to those in the preceding section/

section of this thesis. Most of the tissues examined were obtained at autopsy, within 24 hours of death; occasionally, tissue was obtained at biopsy, e.g. thyroid, synovial tissue. Minor modifications of the technique and protocols of immunisation are given in the Appendix. Non-specific staining was eliminated by absorption with acetone-dried sheep muscle or guinea-pig liver powder. Specificity of staining was demonstrated by the method described previously (pp. 8-11). Thus, pre-treatment with unlabelled anti-human-glomerulus serum inhibited staining, whereas normal rabbit serum had no such effect. Absorption of the labelled antiserum with human kidney, glomeruli or spleen removed the antibody, whereas absorption with rat kidney had no such effect. Species-specificity was investigated as described on p. 11 ; no cross-reaction occurred except with monkey kidney. An antiserum prepared to whole monkey kidney gave a strong cross-reaction with human kidney (see Section III, pp. 136 - 137).

The following human tissues were treated with anti-human-glomerulus globulin:- heart, mesenteric artery, trachea, lung, submandibular gland, oesophagus, stomach, liver, pancreas, kidney, bladder, uterus, uterine tube, placenta, umbilical/

umbilical cord, spleen, lymph node, pituitary, adrenal, thyroid (normal and thyrotoxic), peripheral nerve, skin, skeletal muscle, synovial membrane and intra-articular meniscus.

RESULTS

The pattern of staining obtained with the anti-human-glomerulus globulin in human tissues was essentially similar to that described in the preceding Section, but was much brighter in all tissues examined. A brief report of these results has been presented at a Faraday Society Discussion (Cruickshank & Hill, 1953b).

Basement Membrane

Staining of basement membrane was observed in the trachea, submandibular gland (Fig.23), oesophagus, stomach (Fig.24), pancreas (Fig.25), kidney (Fig.26), bladder, uterus, uterine tube (Fig.27), thyroid (Figs.28-30) and skin. Variation in thickness was again seen in different organs, a very thick membrane being seen around mucous glands at the lower end of the oesophagus and in the uterine tube (Fig.27). Splitting of the membrane to enclose nuclei, such as was seen in the rat thyroid and breast, was noted in the human thyroid but was less marked than in the rat (compare/

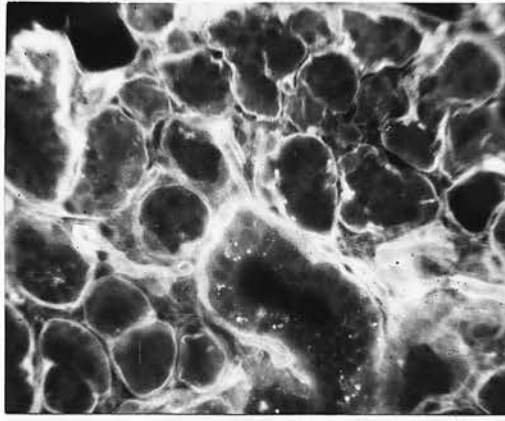


Fig.23. Human submandibular gland. Basement membrane around acini and a duct and capillary reticulin are seen. AG (x 325).

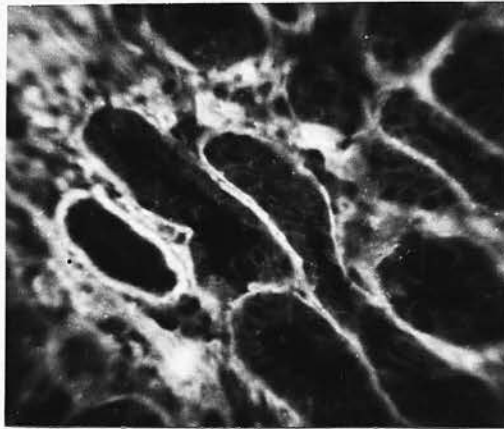


Fig.24. Human stomach (body). A very distinct basement membrane is seen around glands in the mucous membrane. AG (x 325).

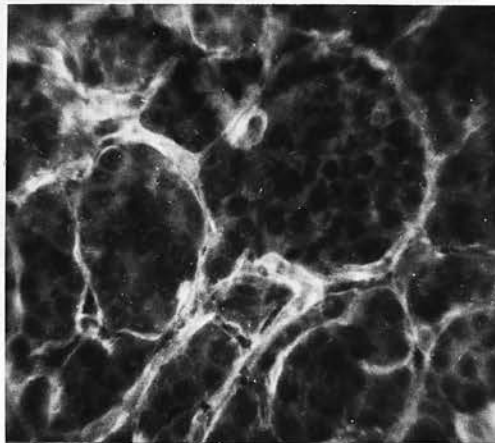


Fig.25. Human pancreas. Acini are surrounded by a basement membrane, and an islet (centre right) is surrounded by a fine reticulin membrane. Reticulin is also seen in a collapsed capillary in the islet. AG (x 325).



Fig.26. Human glomerulus. The basement membrane of the tuft is very clearly seen, that of Bowman's capsule and convoluted tubules less distinctly. AG (x 420).

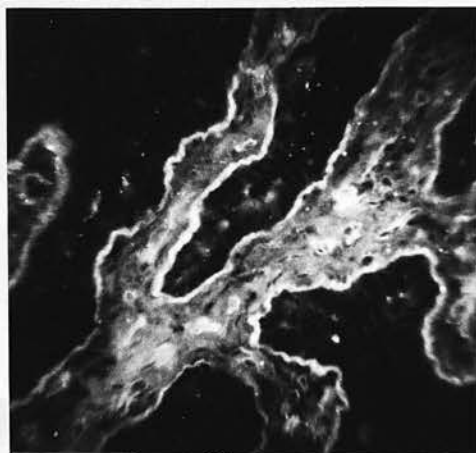


Fig.27. Human fallopian tube. A very thick subepithelial basement membrane is seen. Capillary reticulin is visible in the stroma of the villi, in which there is some non-specific background staining. AG (x 250).

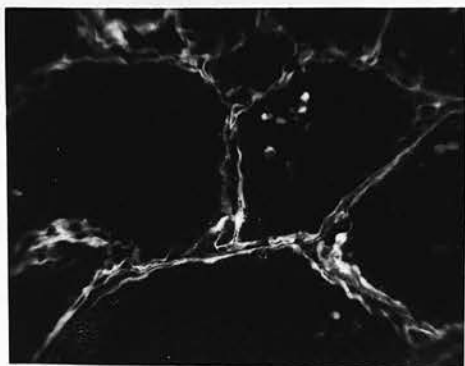


Fig.28. Human thyroid (normal). A fine membrane surrounds the acini and fine strands are also seen between the acini. Differentiation of basement membrane and reticulin is not completely clear in this figure. AK (x 325).

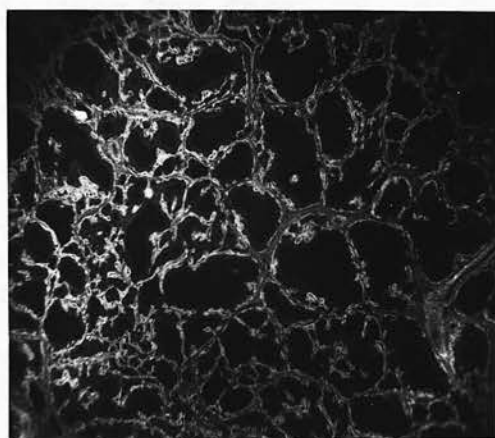


Fig.29. Human thyroid (thyrotoxicosis). Very extensive specific staining is seen in periacinar basement membrane and capillary reticulin. AG (x 50).

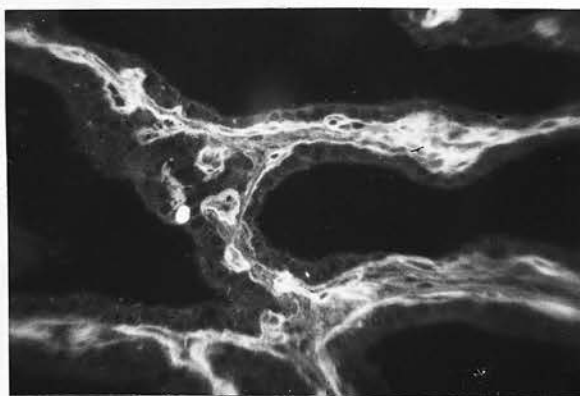


Fig.30. Detail of Fig.29. The very fine periacinar basement membrane is clearly seen. AG (x 325).

(compare Figs. 28 & 12). Reduplication of the membrane, such as was seen in the rat bladder was not present in the human bladder, but was seen in the oesophagus, both under the surface epithelium and around glands in the lamina propria. In the latter situation three or four layers were sometimes seen. Although the membrane in the uterine tube was as thick as that in the oesophagus no reduplication was seen.

In the lung, where difficulty in preparation of sections was again encountered, the appearances suggested that both basement membrane and capillary reticulin in the alveolar walls were stained (Fig.31).

Reticulin

Reticulin was studied in the spleen (Figs.32,33), lymph nodes, liver (Fig.34), pituitary, adrenal (Fig.35), synovial tissue (Fig.36), capillaries in all tissues, including the placenta (Fig.37) and around plain muscle fibres in the oesophagus, stomach, bladder (Fig. 38) and umbilical cord (Fig.39).

In the spleen a membrane was seen around cells both in the corpuscles and in the pulp (Figs.32 & 33); sinusoids in the latter were clearly/

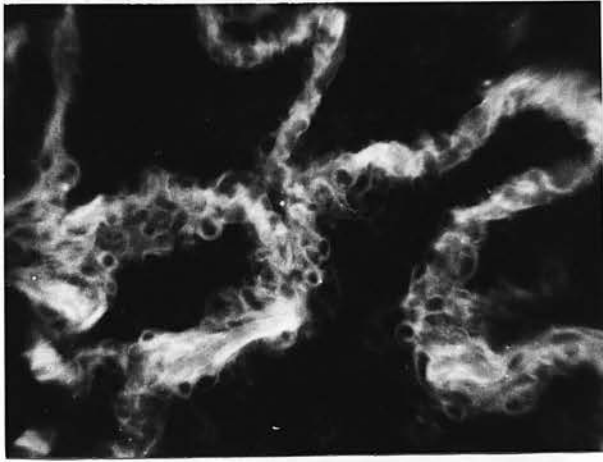


Fig.31. Human lung. There is a suggestion of a basement membrane as well as capillary reticulin. (This was more clearly seen under the microscope for the section is much thicker than the others. The large amount of elastic tissue in the septa also makes this figure difficult to interpret). AG (x 200).

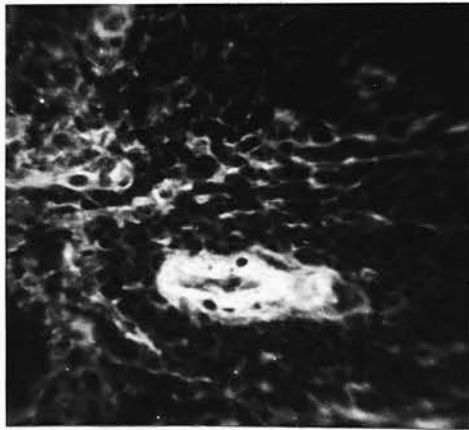


Fig.32. Human spleen. A Malpighian corpuscle showing pericellular reticulin. Specifically stained reticulin laminae are also present in the arteriole but are not clearly distinguishable from the elastic lamina. AG (x 325).

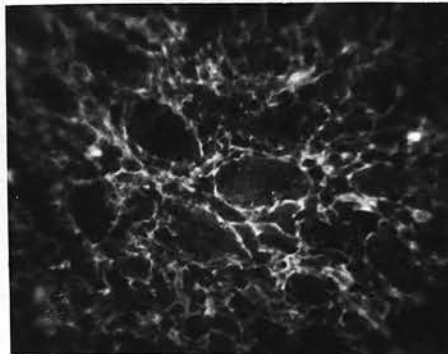


Fig.33. Human spleen. Reticulin surrounds sinusoids and cells in the red pulp. AG (x 480).

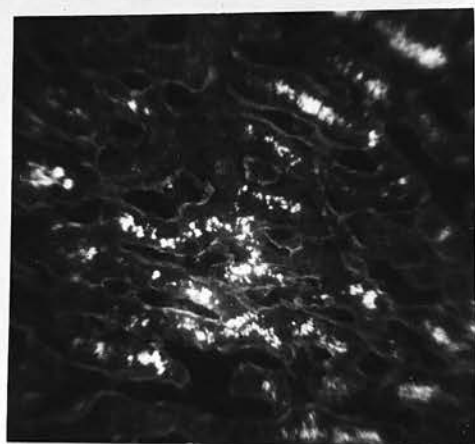


Fig.34. Human liver. Specific staining is present only in the reticulin of the sinusoids. The white globules are ceroid, distinguishable by its bright yellow autofluorescence. (x 325).

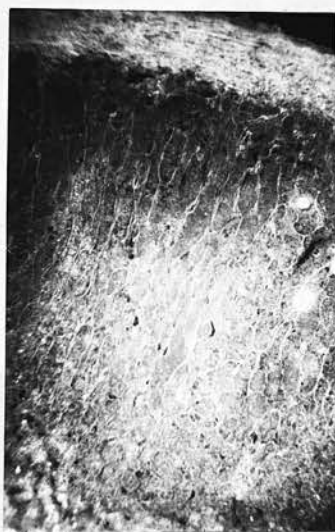


Fig.35. Human adrenal. Fine reticulin fibres surround cell columns and outline sinusoids in the cortex. There is some non-specific staining of cell cytoplasm (as in the rat, Fig.18). Autofluorescence of collagen in the capsule was particularly bright, but no specific green staining was seen there. AG (x 325).

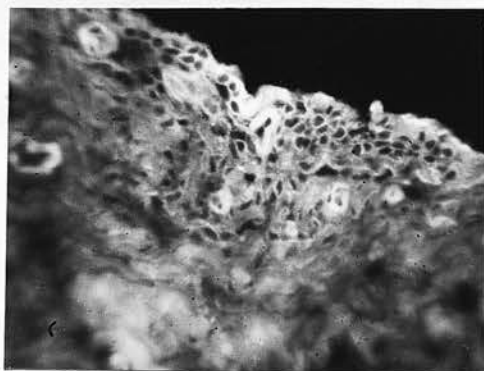


Fig.36. Human synovial tissue. Specific staining is seen in pericellular reticulin fibres and in the walls of capillaries. Bright autofluorescence in collagen beneath the surface. AG (x 250).

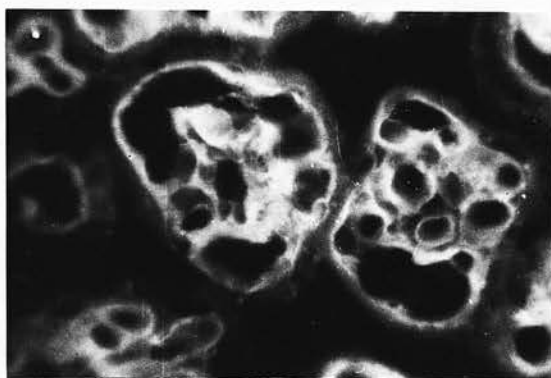


Fig.37. Human placenta. Very bright specific staining of capillary reticulin in two villi. AG (x 480).

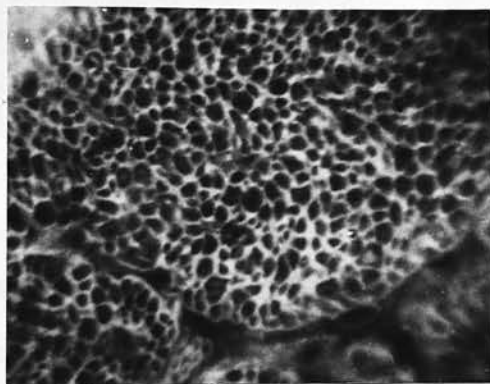


Fig.38. Human bladder. Cross-section of muscle coat showing very bright reticulin around individual muscle fibres. AG (x 400).

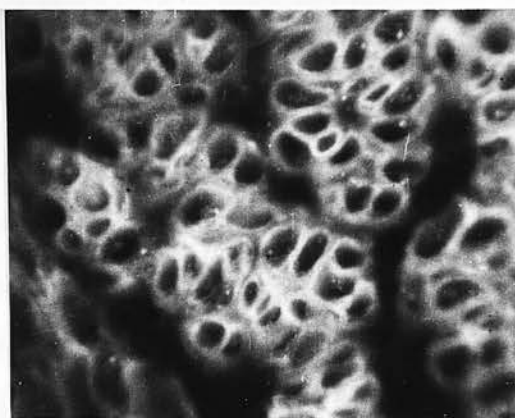


Fig.39. Human umbilical cord. A thick reticulin fibre surrounds muscle fibres in the umbilical artery. AG (x 650).

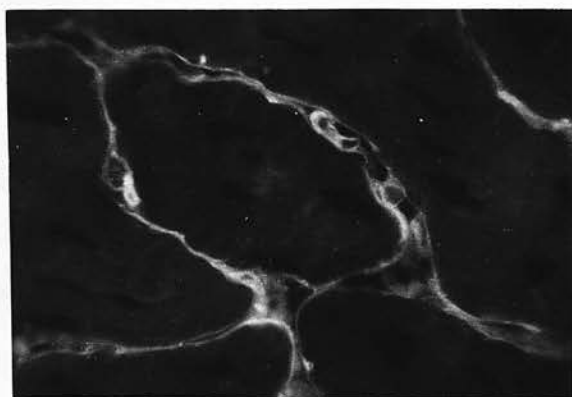


Fig.40. Human skeletal muscle. Specific staining is seen in the fine sarcolemmal sheath and in capillary reticulin between the muscle fibres. AG (x 480).

clearly outlined (Fig.33). Sinusoids in the pituitary and adrenal were also outlined, as in the rat. Sinusoids in the liver were outlined by a thick membrane (Fig.34), a situation where it had been difficult to obtain staining in the rat. In synovial tissue a bright membrane surrounded the synovial cells and reticulin in vessels was also stained; no other staining was seen (Fig.36). Staining in capillaries and larger vessels was of the same distribution as in the rat, a particularly fine result being obtained in placental villi (Fig.37). Reticulin around plain muscle fibres was seen as in the rat. A distinct membrane was observed beneath the mural and valvular endocardium.

Other Connective Tissue Components

Staining of the sarcolemma of striated muscle (Fig.40) and cardiac muscle and of neurilemma was similar to that in the rat. Once again no staining was seen in collagen fibres, elastic fibres, cartilage matrix, or ground substance. The contrast between the green staining of reticulin and the blue autofluorescence of collagen was more striking than in the rat, because of the greater brilliance of the fluorescein-staining in human tissues. Fig.36 shows the appearance/

appearance of specific staining around synovial cells and its absence in the fibrocartilage of the underlying meniscus.

DISCUSSION

Before discussing the main features of these results, it is necessary to define clearly the terms basement membrane, reticulin and collagen as used here. Basement membrane is the fine, argyrophilic structure found around all glandular structures and at other boundaries between epithelium and connective tissue. Reticulin fibres are single, of uniform structure, though varying in diameter, branching, argyrophilic and situated at boundaries within connective tissue, e.g., under vascular endothelium, around fat cells and plain muscle fibres and forming the framework of the reticulo-endothelial system. Collagen fibres occur in bundles, are of uniform diameter, non-branching and react poorly or not at all with silver methods.

The results of the experiments on basement membrane and reticulin will be discussed under two headings:-

- a) basement membrane and reticulin
- b) reticulin and collagen.

Basement Membrane and Reticulin

The results indicate that staining of basement membranes and reticulin fibres throughout both the rat and man can be obtained by treating tissues with a fluorescein-labelled antiserum to the kidney of the same species. Furthermore, the same pattern of staining occurred with an antiserum to isolated glomeruli. Since basement membrane and reticulin components of the glomeruli were not used separately to immunise rabbits, it is important to determine whether the observed reactions were due to separate antibodies to these two tissue components.

The preparations of glomeruli used were not pure, but contained some small portions of proximal convoluted tubule. The absence of specific cellular staining outside the kidney excludes cellular elements of the glomeruli as the source of the widespread connective tissue antigen. The possible extracellular antigens in the glomeruli are the basement membranes of the capillary loops and of Bowman's capsule, both of which were present in the preparations used. The existence of interstitial tissue in the glomerulus - the mesangium of Zimmermann (1933) - is still disputed and is denied by many recent workers. Those who have observed it in electron/

electron microscopic studies have described cells, but no fibres (Mueller et al., 1955; Policard et al., 1955; Yamada, 1955).

The idea of a common antigenicity of basement membrane and reticulin is supported by several observations. First, both reacted similarly with anti-kidney and anti-glomerulus globulins. Second, it was possible to inhibit staining of both elements by absorption of labelled anti-kidney or anti-glomerulus globulins with a homogenate of spleen which contains only reticulin. Third, the experiments with anti-placenta and anti-aorta sera, quoted in the introduction, indicate that antisera to tissues containing reticulin but no basement membrane are nephrotoxic. The present experiments and those already quoted with anti-lung sera cannot be quoted in this argument for both basement membrane and reticulin are present in lung tissue.

There is much evidence derived from histological, biochemical, biophysical and histochemical work which supports the immunological evidence for the identity of basement membrane and reticulin. First, both are argyrophilic. Gersh & Catchpole (1949), using the periodic acid-Schiff/

acid-Schiff technique considered that basement membrane is condensed ground substance and distinct from reticulin, but most of the membranes they studied had an argyrophilic central portion which can be interpreted as reticulin. Leblond (1950), using the same technique observed that the distribution of basement membranes and reticulin fibres in the rat was similar to that shown by silver impregnation. McManus(1950), Rinehart (1955), Bergstrand (1957) & Robb-Smith (1957) deny that the glomerular capillary basement membrane is argyrophilic, but the illustrations of Bohle & Krecke (1955) of rat glomeruli, and of Allen (1951), Jones (1951) & Mueller et al. of human glomeruli indicate that argyrophilia can be demonstrated. Second, both give similar staining reactions with acid-aniline dyes. Third, the work of the Oxford school indicates that the electron microscopic (Little & Kramer, 1952), X-ray diffraction (Little & Windrum, 1954) and metachromatic (Kramer & Windrum, 1954) appearances of basement membrane and reticulin from human renal cortex, liver, adrenal, spleen, lymph node and myocardium are the same. Fourth, Glegg et al. (1953) found that "reticular tissue" from lymph node, lung, testis and adipose tissue all had the same carbohydrate/

carbohydrate components: several of these were identified in renal cortical reticulin by Windrum et al. (1955). Fifth, Brewer (1957), using the polarising microscope, found similar properties in reticulin and basement membrane.

Despite the many resemblances between basement membrane and reticulin, there is evidence that they may not be completely identical. Thus, Lillie (1952a), using the allochrome modification of the periodic acid-Schiff method (Lillie, 1951) found that intralobular reticulin in the liver and follicular reticulin in the spleen were allochroic, similar to collagen, whereas reticulin in vessels, around plain muscle in the oesophagus and in the lung all reacted similarly to basement membrane and were not allochroic. Scott (1957) has repeated the present work with the fluorescent antibody method, using anti-human-glomerulus and anti-human-synovium globulins. His results with direct staining and cross inhibition experiments are of great interest and are reproduced in Table II. Scott suggests that basement membrane and reticulin differ antigenically and that renal glomeruli contain both antigens. However, the results in glomerular capillaries and tubular basement membrane and especially the apparent difference between the reticulin of/

* TABLE II - Results of Direct Staining and Cross Inhibition Experiments

Organ	Histological component	Cross inhibition experiments		
		Direct staining experiments	Anti-glomerulus conjugate	Anti-synovium conjugate
Kidney	Glomerular capillaries	+	-	+
	Glomerular epithelial cells	+	-	-
	Parietal capsule of glomerulus	+	+	-
	Tubular basement membrane	+	+	-
	Tubular cells	-	-	-
	Peritubular capillaries	+	+	-
	Media of arteries	+	-	+
	Acinar basement membrane	+	-	+
	Peri-acinar reticulin	+	-	-
	Interstitium	+	+	-
Thyroid	Capillaries	+	+	-
	Media of arteries	+	-	+
	Reticulin	+	+	-
	Fibrils in trabeculae	+	+	-
Spleen	Media of arteries	+	-	+
	Sarcolemma	+	+	-

* from Scott (1957).

of capillaries and that of the media of arteries do not agree with this interpretation.

There is one further point that is of interest. Although Little & Kramer demonstrated that isolated basement membrane and reticulin have the characteristic periodic banded structure of collagen in the electron microscope (Schmitt et al., 1942), most studies of sections of glomeruli in the electron microscope have not revealed such fibres (Hall, 1954 - rat, rabbit, dog and man; van Breemen et al., 1955 - rat; Mueller et al. - dog and man; Pease, 1955 - rat; Policard et al. - rat; Rinehart - rat; Yamada - mouse; Farquhar et al., 1957 - rat, mouse, rabbit and man). A delicate, fibrillary structure was noted in places in the mouse glomerular basement membrane by Reid (1954) and a lamellated structure by Rhodin (1955), but neither detected any periodicity. Mueller et al. described a lamellated structure in the basement membrane of Bowman's capsule but periodicity is not mentioned. Neither Sjöstrand & Rhodin (1953), in the mouse, nor van Breemen et al., in the rat, were able to demonstrate a fibrillary structure in the basement membrane of renal tubules. Similar results have been described/

described in other tissues, for although Karrer (1955) found fibrils in the basement membrane of the rat lung he was unable to detect any periodicity. The discrepancy between the results of all the other workers and those of Kramer & Little may be explicable by the fact that the Oxford workers used material isolated by maceration with dilute alkali or trypsin, whereas all the others used sections. Pease commented that fibrils may be masked by amorphous material and Karrer that lack of contrast or inadequate resolution may explain failure to demonstrate periodicity. It should be noted that even Kramer & Little found much amorphous material in their preparations. Final morphological identification of collagen fibrils in sections of basement membrane and reticulin may, therefore, be merely a technical matter.

In summary, it can be said that the present work supports other evidence from histological, biochemical, histochemical and electron microscopic studies that basement membrane and reticulin are closely similar, to the extent of possessing common antigenicity. Remarks about reticulin in the following paragraphs are therefore probably applicable also to basement membrane.

Reticulin/

Reticulin and Collagen.

The consistent inability to stain collagen with any of the labelled globulins is interesting. At the time when this work was started, there was a widespread belief that reticulin and collagen were very closely related. Indeed, most authorities believed that reticulin was merely immature collagen (Gross & Schmitt, 1948; Angevine, 1951; Gersh, 1952; Kellgren, 1952; Morrione, 1952; Porter, 1952). This opinion was based on histological and biophysical studies of mature collagen from various sources and "reticulin" fibres from the dermis. Frequently the material was teased out, extracted and reprecipitated, or otherwise isolated from its natural state before study. It is now clear that the "reticulin" fibres so studied were different from reticulin as defined in the present context and that they were immature collagen fibres.

Lillie (1953b) was perhaps the first to draw attention to the fact that the identity of true reticulin, in liver and kidney, and collagen had not been demonstrated. His studies with the allochrome procedure, mentioned above, clearly demonstrated that mature collagen is allochroic whereas basement membrane and most reticulin are not. Subsequently/

Subsequently Little & Kramer have shown in the electron microscope that, although reticulin from various sources contains fibrils which resemble collagen, there is an additional amorphous component in reticulin. This was identified by X-ray diffraction (Little & Windrum) and chemical analysis (Windrum et al.) as a fatty acid, which comprises 11 per cent of the material. Windrum et al. have also shown that the amino-acid composition of reticulin and collagen are similar and that the carbohydrate content of reticulin is 4 per cent, whereas that of collagen is probably negligible. The sulphation-metachromasia technique of Kramer & Windrum, in which sulphuric acid esters of the carbohydrate are produced and demonstrated by metachromasia, confirms this for reticulin becomes metachromatic, whereas collagen remains orthochromatic. These workers also found that treatment with pectinase prevented the metachromasia of reticulin but did not affect its staining with the collagen stain picromethyl blue and they regard this as further evidence that reticulin consists of collagen plus polysaccharide. Glegg et al. also found a higher carbohydrate content in reticulin than in collagen. Brewer's studies on birefringence also indicate that reticulin and collagen fibres are different.

There/

There is thus good histochemical, biochemical and biophysical evidence that reticulin and collagen, although related, differ considerably from one another. The present immunohistochemical studies support this view and indicate that labelled anti-glomerulus serum can be used as a stain for basement membrane and reticulin which gives results as good as those of any other method available and has the additional advantage of the specificity inherent in an immunological reaction. Indeed, it has been possible to demonstrate clearly a basement membrane in several situations where its presence had been doubted. Thus there can be no doubt that a definite membrane surrounds the thyroid acini (compare Figs. 41 & 42 with Figs. 11-13 & 28-30). Lansing & Wolfe (1944) reviewed the literature and reported their own findings with Gomori's silver method. Like earlier workers they regarded all perifollicular reticulin as pericapillary in nature and do not use the term basement membrane. However, Gersh & Catchpole described a very thin basement membrane consisting of fine reticulin fibrils enclosed in Schiff-positive material and Dempsey & Paterson (1955) have described a basement membrane in electron microscope studies. Likewise, a membrane was demonstrated immunohistochemically at the dermo-epidermal junction/



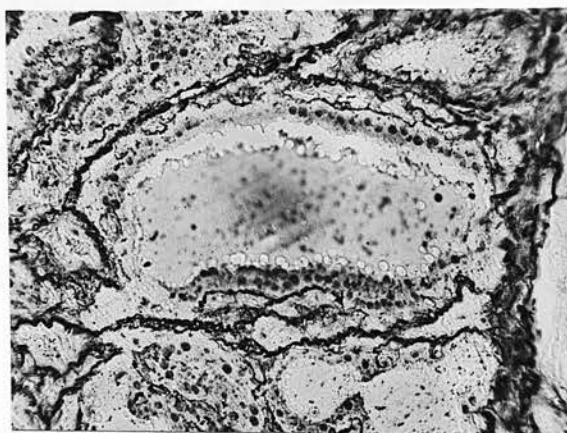


Fig.41. Human thyroid. The basement membrane of the acinus is incompletely impregnated. Reticulin fibres in the interstitial tissue are well seen. Gordon & Sweet reticulin stain. (x 250).

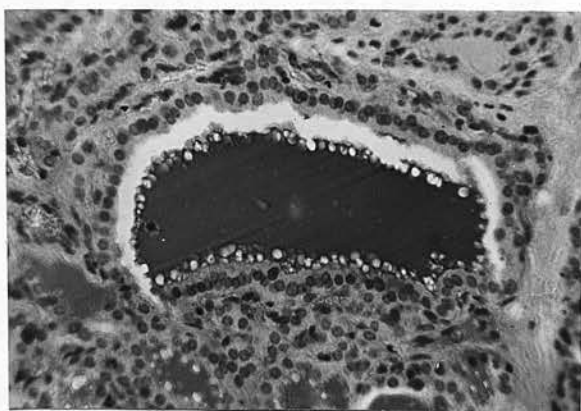


Fig.42. Human thyroid. Serial section to that in Fig.41. The acinar basement membrane is seen with difficulty and only in a few places. Periodic acid-Schiff-haematoxylin (x 250).

junction (Fig.10). Medawar (1953) doubted the existence of a basement membrane in this situation and subsequent electron microscopic (Selby, 1955) and histochemical (Cooper, 1956) work has not revealed the presence of reticulin.

It would clearly be of interest to determine the chemical nature of the antigen(s). The results of Lillie (1952a) and Scott (1957) indicate that the glyco-lipo-protein complex from renal cortical reticulin studied by Windrum et al. may not be the only antigen involved. The lack of effect of hyaluronidase and the absence of staining in cartilage matrix in the present work suggest that neither hyaluronic acid nor chondroitin sulphate is directly involved. However, the immunological difference between reticulin and collagen must be determined by the non-protein components of the former, for both have the same amino-acid composition (Windrum et al.; Bowes et al., 1955). The demonstration by Cole et al.(1951) that a mucoid soluble substance prepared by tryptic digestion of rat kidney could absorb out nephrotoxic antibodies from anti-rat-kidney serum was of great interest, for it suggested that further study of such material might yield useful information about the nature of the glomerular antigen(s). Some of/

of the immunological properties of such digests are discussed later in this thesis (Section III, pp. 81 - 100). The importance of mucopolysaccharides, particularly chondroitin sulphate (Jackson, 1952), in the metabolism of collagen suggested that, although it is not necessarily related to the glomerular antigen(s), an attempt should be made to prepare an antiserum to it. These experiments are discussed on pp. 67 - 80. Finally, it was obviously desirable to investigate the antigenic relationship of reticulin and collagen in more detail. Preliminary attempts to prepare an antibody to collagen are described in the next Section.

SUMMARY

Rabbit antisera to rat kidney, rat glomeruli and rat lung were labelled with fluorescein isocyanate and applied to sections of many rat tissues. All three sera reacted with basement membrane and reticulin throughout the rat and with sarcolemma and neurilemma. The immunological specificity of the reactions was established.

Rabbit antisera to human glomeruli gave a similar pattern of specific reaction in human tissues.

Cross-reactions occurred only with closely related species, thus anti-rat-sera reacted with mouse tissues and anti-human-sera with monkey tissues.

No reaction occurred in either species with collagen fibres, elastic fibres, cartilage matrix or ground substance.

The results are discussed in relation to recent histochemical, biochemical and biophysical work on basement membrane, reticulin and collagen.

It is concluded that basement membrane and reticulin are very closely related, if not identical, and that they show distinct differences from collagen.

COLLAGEN

At the time when this work was started, only three studies of the antigenicity of collagen had been made. Loiseleur & Urbain (1930) immunised rabbits with a solution of rat tail tendons in dilute acetic acid. They obtained complement fixing antibodies, but did not show that these were specific to collagen rather than to other components of the tendons. Battista (1949) /

(1949) was unable to demonstrate antibodies in guinea-pigs or rabbits implanted with "collagen" derived from beef bone by treatment with hydrochloric acid. Waksman & Mason (1949) were unable to demonstrate antibodies in rabbits or guinea-pigs immunised with human collagen alone, coupled to globulin or with adjuvants. They also attempted, unsuccessfully, to produce antibodies in rabbits to rabbit collagen alone or coupled with sulphonic acid. The collagen preparations had been extracted successively with sodium chloride, sodium phosphate and ether and then suspended in absolute alcohol and ground in a ball mill for 48 hours. Waksman & Mason comment that the chemical and physical manipulations used may have affected the antigenicity of the collagen.

The results of the present experiments with anti-sera to glomeruli indicated the desirability of making further attempts to prepare an antibody to collagen. Human collagen was chosen because it was more readily available in quantity and because it was hoped to apply labelled anti-sera to human reticulin and collagen to a study of human pathological material. Purely physical methods were used in the preparation of material for injection in the hope of avoiding any/

any denaturation of the protein, and obtaining the collagen in native or near-native form.

Antigenicity of "Native" Collagen

Adult human patellar tendon, obtained at autopsy, was cleaned as free of muscle and other tissue as possible and stored in the deep freeze. Sections 25 μ thick were cut, in the freezing cabinet and stored, without thawing, in a closed vessel in the deep freeze. Various procedures were used in attempts to get a fine emulsion for injection:

- a) pushing the sections, after thawing, through the fine sieve used to isolate glomeruli (see Appendix, p. 186).
- b) spinning in saline in a Waring Blender for up to 15 minutes; the blender was kept cool in an ice and water bath.
- c) grinding in saline in a ball mill for up to 4 hours.
- d) ultrasonic vibration for 1 hour at 15 watts (performed by Dr. G.G. Selman, Department of Animal Genetics, University of Edinburgh).
- e) grinding with ~~and~~ without sand in a pestle and mortar.

None of these procedures was successful in producing an emulsion suitable for injection, although some fine particles were obtained in c) and d). The saline with the fine particles (approximately 1 gm. solid in 10 ml.) so obtained was separated from the coarser material and stored in/

in the deep freeze. It was used later as antigen in complement fixation tests.

An attempt was made to prepare a powder from small pieces of tendon which had been dehydrated for 48 hours at room temperature in the freeze-drying apparatus (see Appendix, p. 202). The dried tendon could not be pulverised in a mortar.

As physical methods had failed to provide a preparation which could be injected, it was decided to implant sections of tendon at open operation. Two rabbits (53 & 54) received approximately 2.5 gm. of tendon sections intraperitoneally in four doses at intervals of 14-17 days. They were bled immediately before the second and third implantations and 15 days after the fourth implantation. (See Appendix, p.192 for details).

The sera were tested for complement fixing antibodies against diluted saline suspensions of fine particles of the tendon antigen and of homogenised glomeruli by the method of Bedson & Bland (1929) (for details see Appendix, pp. 200-201). The results are shown in Table III. Absorption of the sera with approximately 1 mgm. tendon/ml. abolished the reactions. Precipitin tests/

TABLE III - Complement fixation tests with anti-tendon sera

Antigen	Normal rabbit serum	Rabbit 53			Rabbit 54		
		1st bleeding	2nd bleeding	3rd bleeding	1st bleeding	2nd bleeding	3rd bleeding
Tendon suspension	-	256	16	a/c	a/c	32	a/c
Glomerulus suspension	-	-	-	a/c	a/c	-	a/c

59.

a/c anticomplementary. Results given as
reciprocal of highest dilution of sera
which reacted.

tests were performed with the same sera against human serum and chondroitin sulphate (see p. 69) using the interfacial ring method, with the results shown in Table IV.

One of the sera (Rabbit 53, 3rd bleeding) was then labelled with fluorescein isocyanate, absorbed with sheep muscle powder and applied to sections of human kidney and spleen, but no specific staining was obtained.

Antigenicity of soluble collagen

Shortly after the experiments with human tendon had been completed, Watson et al. (1954) reported the successful production of a specific antibody to rat collagen in rabbits immunised with a solution of tail tendon in dilute acetic acid. These experiments were repeated with the intention of labelling the antiserum with fluorescein and comparing its reactions with those of the antisera to rat basement membrane and reticulin.

Acid-soluble collagen was prepared from rat tail tendon by the method of Watson et al. (see Appendix, p. 189). Electron microscopy of material precipitated from the solution showed the presence of collagen fibrils.

TABLE IV - Precipitin tests with anti-tendon sera

Antigen	Normal rabbit serum	Rabbit 53		Rabbit 54	
		1st bleeding	2nd & 3rd bleedings	1st - 3rd bleedings	
Human serum	-	-	-	-	
Chondroitin sulphate	-	1 [†]	-	-	

† Only partial inhibition of haemolysis.

Four rabbits (150-153) each received 212 mgm. of this 0.1 per cent solution of collagen in 1/10,000 acetic acid in four courses, and five rabbits (160-164) each received 140 mgm. in two courses. The solution was given intraperitoneally in doses of 4 ml. four times a week. Rabbits 150-153 were bled thrice; rabbits 160-164 once (see Appendix, pp. 196 & 197).

The undiluted sera were tested for complement fixing antibodies against the 0.1 per cent solution of collagen in acetic acid. Positive results with rabbits 150-153 were obtained only in the third samples of sera. Serial dilutions of sera were not tested. Those sera which reacted with the collagen were tested for precipitating antibodies against rat serum. The results are shown in Table V.

The experiments had to be stopped at this point.

DISCUSSION

The results of these two experiments cannot be regarded as conclusive, but they are encouraging. The human tendon material was used, in a form as little altered from the native state as possible, so that, although the collagen was undegraded/

TABLE V - Tests with anti-collagen sera

Antigen	Rabbits					
	150 [†]	151 [†]	152 [†]	153 [†]	160	161 162 163 164
Collagen	±	++	-	+	-	+ - - -
Rat serum	-	-		-		- - - -

[†] Third sample of serum; previous two samples negative.

undegraded, other extraneous and potentially antigenic substances were probably present. Serological analysis of the antisera has excluded the presence of antibodies to basement membrane, serum proteins or to chondroitin sulphate. It is therefore possible that a specific antibody to collagen was present.

It was hoped that studies with fluorescein-labelled serum would help to prove this, but these experiments gave negative results. This may well have been due to the antibody being present in so low titre that it was lost completely on conjugation (see Section V, p. 166). The titre of the present sera was not determined after conjugation, but it is possible that this procedure itself may have been responsible for the lack of staining. This difficulty might be overcome by the indirect method (see p. 166), but, unfortunately, no anti-rabbit globulin serum was available when these experiments were performed.

It is doubtful whether the use of adjuvants would increase the titre of antisera to insoluble collagen for Watson et al. were unable to increase the titre of antibodies to soluble collagen by using this method. It is possible that the low titre of the serum and particularly the/

the reduction in titre between first and second bleeding of rabbit 53 was due to the presence of an excess of antigen. Large masses of partially necrotic tendon were found in the peritoneal cavity of both animals when they were killed.

The experiment with soluble collagen had to be stopped before it was completed so that no attempt was made to establish the specificity of the antisera other than to exclude antibodies to rat serum proteins. The antigen used was prepared by a method closely similar to that of Watson et al. and was shown electron microscopically to contain collagen. Watson et al. proved by immunological tests coupled with enzymatic digestion that their antisera contained specific antibodies to collagen rather than to tissue proteins or polysaccharides. They also demonstrated that both purified and native rat collagens completely absorbed the antibodies from the sera. Once again, it is possible that a specific antibody to collagen was present in the antisera produced in the current experiments.

It is obvious from the present experiments and those of Watson et al. that collagen is a weak antigen: the titres given by the American/

American workers are for antigen dilution, so that the antibody content of their sera cannot be assessed. Nevertheless the potential value of an antiserum is such that further attempts should be made to obtain it. Recent work on soluble forms of collagen (reviewed by Jackson, 1957) indicates that material extracted by neutral salts, rather than that extracted by acetic acid, is the precursor of insoluble collagen which is the form found in mature fibrous tissue. Humphrey (1956) has found neutral-salt-soluble collagen from rat tendon to be antigenic. Preliminary attempts made by the writer to obtain neutral-salt-soluble collagen from human neonatal skin have not been very satisfactory. Watson (1955) referred to the difficulty of extracting human collagen. It is planned to use neonatal dura mater and fascia lata for extraction experiments in the hope that these almost pure fibrous structures will be more satisfactory than skin.

SUMMARY

Rabbits were immunised by intraperitoneal implantation of untreated sections of human patellar tendon. Their sera contained complement fixing antibodies which reacted with tendon, but not with glomeruli, serum or chondroitin sulphate.

Rabbits/

Rabbits immunised with acid-soluble collagen from rat tail tendon produced antibodies which reacted with acid-soluble collagen, but not with serum.

It is possible that specific antibodies to collagen were produced in both experiments.

CHONDROITIN SULPHATE

In September 1952, when these experiments were started, there was no direct information about the antigenicity of chondroitin sulphate. Elliott (1914) & Goodner (1925) studied the antigenicity of several mucins, including material extracted from cartilage and tendon with strong alkali. The extracts were antigenic, but their exact chemical nature is not stated. Ropes et al (1947) obtained relatively strong precipitin and complement fixation reactions after injecting bovine cartilage (? tracheal) into rabbits. The sera obtained reacted weakly with bovine synovial fluid but not with mucin prepared from that fluid. Biochemical studies, reviewed by Meyer (1951), had established the existence of several different chondroitin sulphates in the tissues and although it was assumed that cartilage cells were the source of that variety found/

found in cartilage (Gardner, 1950) this had not been proven.

It appeared, therefore, that, if chondroitin sulphate were antigenic, studies with fluorescein-labelled antisera might yield useful information about its site of formation and distribution in the normal tissues and perhaps also about changes in pathological conditions. It was decided to use whole human cartilage for immunisation and so avoid extraction procedures which might denature, or depolymerise the mucopolysaccharide; it was hoped that unwanted antibodies could be removed by appropriate absorption tests. Preliminary experiments with whole cartilage had been unsatisfactory when Glynn & Holborow (1952 a & b) published their observations on the conversion of polysaccharide haptens, including human chondroitin sulphate, to full antigens by immunising rabbits with a vaccine of streptococci on to which the polysaccharides had been adsorbed. This technique was adopted forthwith and the results are now described.

METHODS/

METHODS

Two preparations of chondroitin sulphate were used, one prepared from bovine trachea by the method of Bray et al. (1942) and supplied by The Evans Biological Institute, Runcorn, Cheshire, the other prepared from human costal cartilage by Dr. A.B. Roy, Biochemistry Department, University of Edinburgh, using the method of Einbinder & Schubert (1950). The human material had a protein content of approximately 5%. The costal cartilages used were obtained at autopsy from two patients whose blood groups are unknown. Two further preparations of chondroitin sulphate from individuals of blood groups A and O were prepared and supplied to Professor W.T.J. Morgan, Lister Institute. Vaccines were prepared by exposing a culture of Group-A, Type-4 β - haemolytic streptococcus (N.C.T.C. Catalogue No.8326) to solutions of the polysaccharides as described by Glynn & Holborow (1952b). (For details, see Appendix, pp. 187).

Two rabbits (66 & 67) were immunised with divided intravenous doses of vaccines prepared from the bovine, and four (72-75) with vaccines/

vaccines prepared from the human polysaccharide. Injection schedules are given in the Appendix (pp.192-193). The animals were bled 12 days after the final injection and their sera titrated by the capillary tube method of Swift et al. (1943) against chondroitin sulphate, hyaluronic acid (prepared from human umbilical cord and supplied by The Evans Biological Institute), and a Lancefield extract of the streptococci and by the interfacial ring method against normal human serum and a tryptic digest of human kidney (see Section III, pp. 82-83). One of the antisera to human chondroitin sulphate was conjugated with fluorescein isocyanate and used to treat fresh-frozen sections of kidney, trachea and articular cartilage.

Four rabbits (102,103,142,143) were immunised with divided intravenous doses of human chondroitin sulphate solution only and bled 12 days after the final injection. Injection schedules are given in the Appendix (pp.194 & 196). The sera were titrated as above and also by the interfacial ring method against purified blood group substances A, B, H & Le^a (supplied by Professor W.T.J. Morgan). A pooled sample of these sera was also tested against various antigens by a modification of the gel-diffusion method of Ouchterlony//

Ouchterlony (1949) (see Appendix, p. 200). One of these antisera was conjugated with fluorescein isocyanate and used to stain fresh-frozen sections of kidney, trachea and articular cartilage.

RESULTS

The results of precipitin reactions with the sera of the animals injected with the chondroitin sulphate-vaccines are seen in Table VI. No reactions occurred with the sera from animals injected with the preparation from bovine trachea. Anti-sera to human chondroitin sulphate-vaccine reacted with the polysaccharide alone and the Lancefield extract of the organism used to prepare the vaccine. The reaction with hyaluronic acid is probably of no significance, for it is known that this substance frequently gives rise to a precipitate when titrated against serum (Ropes et al.).

All four of the rabbits immunised with chondroitin sulphate alone produced sera which reacted with the polysaccharide (Table VII). Reactions also occurred with a tryptic digest of kidney, but not with human serum. A pooled sample of these sera reacted with blood group substances A,B & H, so the tests were repeated after aliquots of the sera had been absorbed with 1 mgm. blood group substance per ml. The results of/

TABLE VI - Titration of antisera to bovine and human chondroitin sulphate-vaccine

Antigen	Normal rabbit serum	Bovine CSO ₄ -vaccine		Human CSO ₄ -vaccine	
		66	67	72	73 74 75
Bovine chondroitin sulphate	-	-	-	-	-
Human chondroitin sulphate	-	-	-	2	2
Hyaluronic acid	-			+	
Heparin	-			-	
Human serum	-			-	
Kidney digest (4)	-			-	
Lancefield extract	-	+		+	

CSO₄ Chondroitin sulphate.

TABLE VII - TITRATION OF ANTISERA TO HUMAN CHONDROITIN SULPHATE

Antigen	NRS	102		103		142		143	
		Serum titre	Antigen titre	Serum titre	Antigen titre	Serum titre	Antigen titre	Serum titre	Antigen titre
Human CSO ₄	-	16	10,000	4	10,000	4	6,400	4	1,600
NHS.	-	-	-	-	-	-	-	-	-
Kidney digest (7)	-	4	1	1	4	4	1	4	1

NRS Normal rabbit serum
 CSO₄ Chondroitin sulphate
 NHS Normal human serum

of the tests before and after absorption are shown in Table VIII. The result obtained in the gel-diffusion test with this serum against human chondroitin sulphate, tryptic digest of glomeruli and blood group substances is seen in Fig.43 - a reaction of identity with all the antigens.

Further tests with this pooled serum were performed by Professor Morgan, who found:

- a) no precipitation when undiluted serum was titrated with blood group substances at dilutions of 1/1000 or higher. Anti-sera prepared to blood group substances give a distinct precipitate at 1/10,000 and frequently at 1/100,000.
- b) haemagglutination by the serum of human erythrocytes to titres of 1/16 (Groups A & O) or 1/64 (Group B.). These values occur with normal rabbit sera.
- c) no evidence of blood group activity in the original preparation of human chondroitin sulphate or in the preparations from individuals of known blood group.

Antisera to the vaccine-treated and untreated chondroitin sulphate were labelled with fluorescein isocyanate and tested against human articular cartilage, trachea and kidney. No specific staining was obtained.

DISCUSSION

The methods employed for the extraction of tissue polysaccharides have been reviewed recently by Snellman (1957) who points out that the/

TABLE VIII - Precipitin tests with blood group substances

Serum absorbed with	Chondroitin sulphate	A substance	B substance	H substance	Le ^a substance
-	++	++	++	+	-
A substance	-	-	+	-	"
B substance	+	-	-	-	"
H substance	+	-	+	-	"

Sera used neat, antigens diluted 1/500.

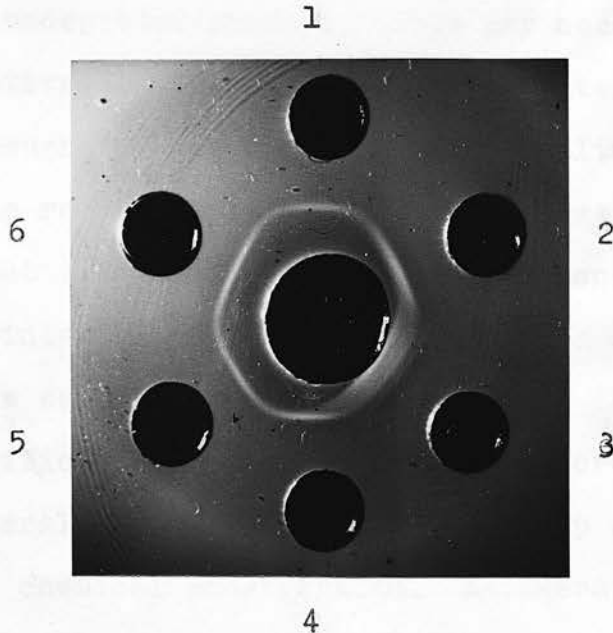


Fig.43. Ouchterlony plate. The centre cup contains anti-chondroitin sulphate serum. The peripheral cups contain chondroitin sulphate (1), blood group A substance (2), blood group B substance (3), blood group H substance (4), blood group Le^a substance (5) and a tryptic digest of glomeruli (6). Photographed after 48 hours incubation at 37°C. Note reaction of identity with all six antigens. The broadening of the band between serum and cup 3 is an artefact.

the alkali method used by Bray et al. gives a degraded product whereas the potassium chloride method of Einbinder & Schubert is milder and may give an undegraded product. This may account for the negative results with the bovine material used here, though the experiments were very limited and cannot be regarded as conclusive. There is no doubt that the human material used was antigenic, but the interpretation of the results is difficult. There are several reasons for regarding them as non-specific. Firstly, the antiserum cross-reacted with several highly purified blood-group substances of known chemical constitution. Antisera to these substances do not cross-react with one another (Aminoff et al., 1950; Annison & Morgan, 1952a & b; Gibbons & Morgan, 1954). Secondly, the tests performed by Professor Morgan indicate that blood group substances were not present in the chondroitin sulphate in detectable amounts and that the reactions with red cells are similar to those of normal rabbit serum. Thirdly, the gel-diffusion test gave only a single line with the reaction of identity (Ouchterlony, 1953) with all the antigens tested. It seems likely, therefore, that even the extraction method of Einbinder & Schubert gives a degraded product from an immunological standpoint.

The human material used in the present experiments has not been analysed chemically, so that the nature of the antigen is not known. Nevertheless the procedure of Einbinden & Schubert was followed exactly so that chondroitin sulphate was presumably present. Glynn & Holborow (1952b) claimed that human chondroitin sulphate prepared by this method was not antigenic in doses similar to those used here, but could be made fully antigenic by adsorption on to streptococci. Streptococci had no adjuvant effect in the present experiments, presumably because the protein present in the preparations was antigenic. Boake & Muir (1955) were unable to repeat the results of Glynn & Holborow, using rabbit chondroitin sulphate, but otherwise following exactly the technique of Glynn & Holborow. Wilson (1955) & Lominski (1957) have also repeated the experiments of Glynn & Holborow, with negative results. Glynn & Holborow (1955) have subsequently stated that their antiserum probably contained antibody to blood group A substance, rather than to chondroitin sulphate. Thus, the antigenicity of chondroitin sulphate remains to be demonstrated.

The original purpose of this work was to use fluorescein-labelled antisera to study the site of formation and distribution of chondroitin sulphate/

sulphate in the tissues. Since the work was started, ^{35}S and labelled sulphate has been used extensively in animal experiments and has given much information on the distribution of sulphated mucopolysaccharides. Thus, Curran & Kennedy (1955a) have injected ^{35}S as sulphate ion into mice and studied its distribution in many tissues and Curran & Gibson (1955-56) have described an in vitro method for use with human tissue. There can be no doubt from this work that the chondroitin sulphate of cartilage is produced in chondrocytes. The origin of the polysaccharide in other tissues has not been determined, for the technique does not differentiate chondroitin sulphate from other sulphated mucopolysaccharides, such as heparin. The granules demonstrable in proliferating fibroblasts are of particular interest in this respect: they have been interpreted by various workers as glycoprotein (Gersh & Catchpole, 1949), hyaluronic acid (Curran, 1953), collagen (Robbins et al., 1955), acid mucopolysaccharide plus protein (Jackson, 1955) and sulphated mucopolysaccharide (Curran & Kennedy, 1955b). A specific and practicable method for demonstrating chondroitin sulphate in human tissue is not yet available. Several new methods for the/

the isolation of chondroitin sulphate have been described recently (Scott, 1956; Partridge & Davis, 1958; Muir, 1958), one of which yields the polysaccharide in a complex containing non-collagenous protein. Further immunological studies with these preparations may provide a specific method for studying chondroitin sulphate distribution in human tissues.

SUMMARY

Rabbits were immunised with chondroitin sulphate extracted from bovine tracheal cartilage with alkali and from human costal cartilage with potassium chloride. Injection of the latter gave rise to antibodies which reacted not only with the original antigen but also with a tryptic digest of human kidney and with several purified blood group substances.

The antigenic component of the preparation has not been identified. Protein was present but not blood group substances. The serological reactions are regarded as non-specific and probably due to degradation of the chondroitin sulphate during preparation.

The results are discussed in relation to other work on the antigenicity of chondroitin sulphate/

sulphate and the use of ^{35}S for the demonstration of sulphated mucopolysaccharides. The need for a specific method for the identification of individual substances is emphasised.

DEVELOPMENTAL AND HISTOCHEMICAL

STUDIES OF THE SPINE

AND BLIND TISSUE

SECTION III.

HUMAN KIDNEY AND GLOMERULI

The evidence which indicates that the glomerulus is the source of the nephrotoxic antigen has been reviewed briefly earlier in this thesis (Section II, pp. 5 - 7). When Cole et al. (1951) demonstrated that trypsin digestion of rat kidney yielded a soluble substance which could induce the kidney-damaging antibody from a

IMMUNOLOGICAL AND IMMUNOHISTOCHEMICAL

STUDIES ON THE KIDNEY

AND BLOOD VESSELS

First, chemical analysis of the digest should reveal the composition of the nephrotoxic antigen which appeared to be identical with the basement membrane-antigen antigen studied in Section I of this thesis (Hill & Grubb, 1950). Second, digestion of human kidney or glomeruli should yield suitable preparations with which to look for these antigens in electron microscopy. Third, an anti-glomerular serum should be prepared for the *in vitro* titration of nephrotoxic sera.

It was decided therefore a) to study the immunological properties of digests prepared from human kidney and glomeruli, with a view to confirming the work of Cole et al. and establishing the

IMMUNOLOGICAL PROPERTIES OF TRYPTIC DIGESTS OF
HUMAN KIDNEY AND GLOMERULI

The evidence which indicates that the glomerulus is the source of the nephrotoxic antigen has been reviewed briefly earlier in this thesis (Section II, pp. 5 - 7). When Cole et al. (1951) demonstrated that tryptic digestion of rat kidney yielded a mucoid substance which could absorb the kidney-damaging antibody from a nephrotoxic serum it seemed possible that the antigen had been obtained in soluble form. This would be an important advance for several reasons. First, chemical analysis of the digest might reveal the composition of the nephrotoxic antigen, which appeared to be identical with the basement membrane-reticulin antigen studied in Section I of this thesis (Hill & Cruickshank, 1953). Second, digests of human kidneys or glomeruli might be suitable preparations with which to look for tissue antibodies in glomerulonephritis. Third, a soluble antigen would be available for the in vitro titration of nephrotoxic sera.

It was decided therefore a) to study the immunological properties of digests prepared from human kidneys and glomeruli, with a view to confirming the work of Cole et al. and establishing the/

the origin of the digests immunochemically, and b) to use the digests, along with other preparations, in an investigation of the role of kidney auto-antibodies in acute glomerulonephritis.

METHODS AND RESULTS

Six extracts of normal human glomeruli and three of whole kidney were prepared by Dr. A.B. Roy, Department of Biochemistry, University of Edinburgh. The kidneys were obtained at autopsy, within 24 hours of death, from patients who gave no history of renal disease; the normality of the kidneys was checked histologically before they were used. The organs were stored at 0°C. until used. The procedure for isolation of glomeruli was a modification of that described by Greenspon & Krakower (1950) (see Appendix, pp. 186-187). In the early preparations, the original method of Cole et al. was used and only small quantities were prepared. Later the method was modified to include deproteinising of the tryptic digests by Sevag's method. Later still, the glomeruli or whole kidney were extracted successively with 0.15M sodium chloride, 1.5M sodium chloride and 5M potassium chloride before digestion, in the hope of removing as much polysaccharide as possible. Larger quantities of the later digests were/

were prepared, including one (digest 7) from 180 gm. of glomeruli which yielded 120 ml.; this preparation contained 0.8% total solid and 0.5% protein. Full details of the final method of preparation are given in the Appendix (pp. 187).

The experiments carried out with these extracts will be discussed under the following headings:-

- a) titration against antisera to whole kidney or glomeruli.
- b) absorption of specific antibody from anti-glomerulus serum.
- c) immunisation of rabbits.
- d) tissue localisation experiments.

Titration against antisera to kidney or glomeruli.

The main property of the extract described by Cole et al. was its ability to precipitate nephrotoxic antibody from anti-kidney serum, so that animals injected with the treated serum did not develop nephritis. Such an experiment was impossible with human material, so the extracts were tested, in the first place, for their reaction with rabbit anti-human-kidney or anti-human-glomerulus serum. The sera included those shown previously to react with basement membrane and reticulin (Section II, pp. 29 - 30) and four/

four further antisera to kidney (Rabbits 89 & 90) or glomeruli (Rabbits 92 & 169) (see Appendix, pp. 193 & 197). The reactions with the immune sera were controlled by tests with normal rabbit serum. Precipitin tests were performed by the interfacial ring method, using serial four-fold dilutions of the extracts layered on top of neat serum. The tubes were stood for 1 hour at room temperature before reading. The results are shown in Table IX. Complement fixation tests, using the technique described previously (Section II, pp. 58) gave similar results.

These experiments indicate that the digests contain a substance which precipitates with antisera to human kidney or glomeruli. It was next necessary to demonstrate the specificity of the reaction.

Absorption of Specific Antibody from Anti-glomerulus Serum.

No reaction occurred in precipitin tests between digests and the antisera to human chondroitin sulphate or tendon described previously (Section II). Some of the earlier digests reacted with antisera to human serum, but this did not occur with digests prepared by the final method. Furthermore, the anti-kidney and anti-glomerulus sera still reacted with the digests after/

TABLE IX - Titration of digests against antisera to kidney and glomeruli

Serum	Rabbit No.	Digests prepared from									
		Glomeruli					Whole kidney				
		3	4	6	7	8	13	9	11	12	
Normal rabbit serum		-	-	-	-	-	-	-	-	-	
Anti-human-glomerulus	48	++	2	++	+++		+++				
	52	+									
	82			±	16						
	169*				++		+	++		+	
Anti-human-kidney	89			++		16					
	90			64	64	16					
Anti-human-glomerulus digest	92				64		16		+		16
	96				256			64			

* Previously immunised with kidney digest (9) in Freund's adjuvants (see p. 197)

after absorption with human serum. The reaction between digests and anti-kidney or anti-glomerulus sera is thus not due merely to the presence of serum proteins in the digests and antibodies to serum proteins in the anti-kidney or anti-glomerulus sera.

Anti-glomerulus serum was absorbed with a wet packed suspension of human glomeruli, allowing the preparation to stand for 1 hour at room temperature and then overnight at 4°C . This procedure has been shown to remove specific anti-glomerular antibodies (Section II, pp. 30). When the absorbed serum was tested for precipitation with the digests, no reaction occurred. Equal parts of labelled anti-glomerulus serum and two of the glomerular digests were mixed, stood for 1 hour at room temperature and overnight at 4°C . and then spun. Drops of the supernates were used to treat sections of kidney. No staining was obtained, whereas no diminution in the intensity of staining occurred when the same serum was similarly treated with phosphate buffer. The reaction between digests and antisera are thus due to a specific antigen-antibody reaction. It was next necessary to demonstrate whether the digests were full antigens or merely haptens.

Immunisation of rabbits.

Two rabbits (78 & 79) were given 3.0 ml. of one of the earlier glomerular digests (No.4) in divided intravenous doses (see Appendix, p.193) and bled 9 days later. The results of precipitin tests, using sera and digests undiluted are seen in Table X. Both rabbit sera reacted with human serum as well as with the digests so the tests were repeated after absorption of the rabbit sera with human serum. A reaction was still obtained with one of the rabbit sera and one of the digests. It seemed possible, therefore, that the digests might contain a full antigen of glomerular origin. Two rabbits (80 & 81) were then immunised intravenously with a streptococcal vaccine similar to that described previously (Section II, pp. 69 - 70) but incubating the organisms in the glomerular digest, in the hope of enhancing its antigenicity. Details of the immunisation schedule are given in the Appendix, p. 193 . The results of precipitin tests (Table X) confirm that the digest used was antigenic. Ouchterlony plates were also used, giving only a single line in the reaction between the digest and the latter two sera.

These/

TABLE X - Precipitin tests with antisera to glomerulus digest (4)

Antigens	Serum Absorbed With	Normal Rabbit Serum	Extract Rabbit 78	Extract alone Rabbit 79	Extract-vaccine Rabbit 80	Extract-vaccine Rabbit 81
Glomerulus digest (3)	-	-	+	+		
" " human serum	human serum		-	-		
Glomerulus digest (4)	-	-	+	++	++	+++
human serum	human serum		-	+		
Human serum	-		+	+	-	-
" " human serum	human serum		-	-		

Sera used undiluted in all tests.

These experiments were repeated with a later digest (No. 7). Three pairs of litter-mates (91 & 92, 93 & 94, 95 & 96) were used, one of each pair being injected with the extract alone (7.5ml. of extract), the other with a streptococcal vaccine prepared from the extract (7.5 ml. of vaccine prepared from 1.16 ml. of extract). All injections were intravenous. Details of the immunisation schedules are given in the Appendix, p. 194. All animals were bled 12 days after the final injection and precipitin tests performed with serial dilutions of sera against digest diluted 1/10 and human serum. Results are shown in Table XI and confirm that the digests contain a full antigen of glomerular origin. It appears that the streptococci had no enhancing effect on the antigenicity of the digest, but as there is a considerable difference in the amounts of digest used in the two groups of animals, further experiments were performed.

Three rabbits (99 - 101) were immunised intravenously with 1.2 ml. of the same digest in divided doses. Two further rabbits (97 & 98) were injected intravenously with a vaccine prepared by incubating the streptococci in saline instead of in the digest; the doses used were the same as/

TABLE XI - Precipitin tests with antisera to glomerulus digest (7)

Antigen	Normal rabbit serum	Extract alone (7.5 ml.)			Extract-vaccine (1.16 ml. extract)				Extract alone (1.2 ml.)			
		Rabbit 92	Rabbit 94	Rabbit 96	Rabbit 91	Rabbit 93	Rabbit 95	Rabbit 99	Rabbit 100	Rabbit 101		
Human glomerulus digest (7)	-	16	16	16	1	1	1	1	1	1		
Saline extract of human kidney #	-	+	+	+	-	-	+	+	+	-		
Human serum	-	-	-	-	-	-	-	-	-	-		
Human chondroitin sulphate †	-	+	+	+	-	-	-	-	-	-		
Rat kidney digest	-	-	-	-	-	-	-	-	-	-		

* Sera used undiluted

† Sera used undiluted; chondroitin sulphate diluted 1/1000.

as in rabbits 91, 93 & 95. All of these rabbits were bled 12 days after the final injection and the sera tested as above. Results with rabbits 99 - 101 are shown in Table XI; sera 97 & 98 did not react with any of the antigens but gave a strong reaction with a Lancefield extract of the organisms. The fact that the titres in rabbits 99 - 101 are the same as those in rabbits 91, 93 & 95 and that the organisms alone produced no anti-bodies to the digest confirm that the streptococci had no adjuvant effect. Furthermore, when the digest was tested against anti-kidney or -digest sera before and after incubation with streptococci, no difference was noted in the reaction (Table XII).

Two rabbits (169 & 170) were immunised with a digest from whole kidney (9) in Freund's adjuvants (Freund & McDermott, 1942) in the hope of increasing the antibody titre. Details of the immunisation schedule are given in the Appendix (p. 197). The total amount of digest given was 1.26 ml./rabbit. The animals were bled 36 days after the final injection. Both rabbits then received glomerulus digest (7) 1 ml. subcutaneously followed by 1 ml. intravenously the next day and were bled 14 days later. Results of tests with these sera are seen in Table XIII.

The/

TABLE XII - Effect of streptococci on precipitin titre of glomerular digest (7).

Glomerular Digest	Anti-kidney serum (Rabbit 90) *	Anti-digest serum (Rabbit 96)
Before incubation	+	256
Incubated 1 hour at 37°C. without streptococci	+	64
Incubated 1 hour at 37°C. with streptococci	+	64

* Serum used undiluted.

TABLE XIII - Precipitin tests with antisera to kidney digest (9).

Antigen	Normal rabbit serum	Rabbit 169		Rabbit 170	
		1st bleed	2nd bleed	1st bleed	2nd bleed
Kidney digest	-	16	-	16	1
Glomerulus digest	-	16		16	-
Human serum	-	-		-	

The adjuvants apparently had a slight effect.

Table XI shows also the results of precipitin reactions between anti-digest sera and two other preparations, namely, chondroitin sulphate and a digest prepared from whole rat kidney. In view of the results obtained in the experiments with chondroitin sulphate described in Section II (pp. 67 - 80), it is likely that the reaction now obtained is of no significance. The lack of cross-reaction with the rat kidney digest is in keeping with the results described previously with antisera to rat or human glomeruli (Section II, pp. 11 & 30).

Tissue localisation experiments.

Three sera (79, 80 & 81) were tested for ability to inhibit the reaction obtained with anti-human-glomerulus serum and two (92 & 96) were labelled with fluorescein and tested on sections of kidney. The results of all these experiments were negative.

DISCUSSION

These experiments indicate that the digests, whether prepared from isolated glomeruli or whole kidney contained antigenic materials. Crude preparations contained serum proteins, whereas/

whereas purer preparations contained species specific glomerular antigens only. Cole et al. mentioned preliminary experiments which indicated that the material was not antigenic, though they had demonstrated that it was haptenic. Yagi et al. (1956) confirmed the haptenic property of digests from rat kidney by demonstrating their ability to decrease the tissue localising activity of ^{131}I -labelled rabbit anti-rat-kidney sera. Electrophoretic analysis indicated the presence of five components. Two of these, which were removed by digestion with ribonuclease and deoxy-:ribonuclease had little effect, whereas the other three, which consisted primarily of protein, had a much more marked effect in decreasing localisation. Immunisation experiments were not reported. These results were confirmed by Goodman & Baxter (1956) who also found the digests to be weakly antigenic in rabbits and the resulting sera to have weak nephrotoxicity. Glynn & Holborow (1952b) found that rabbit antisera to a streptococcus-rat kidney digest-vaccine were nephrotoxic. Liu et al. (1957) found that rabbit antisera to rat kidney digest were antigenic but not nephrotoxic, whereas streptococcus-digest vaccine produced higher titre sera which were nephrotoxic. The differences between these and the present results may be explained/

explained, at least partly, by differences in the amount of antigen injected. Thus Liu et al. gave digest equivalent to 7.9 gm. of kidney, whereas in the present experiments the highest titres were obtained with amounts equivalent to 10.75 gm. of isolated glomeruli.

It is difficult to account for the failure to increase the antibody titre with streptococcal vaccine in the present experiments. However, it should be noted that Glynn & Holborow did not state whether their digest itself was antigenic and they believe that the effect of streptococcal vaccines is due to adsorption of haptenic polysaccharides on to the bacterial particles (Glynn & Holborow, 1952a). It seems unlikely that the digests contain any significant amount of polysaccharide, as their activity is not significantly altered by treatment with hyaluronidase, β -glucuronidase, α -amylase, β -amylase, pectinase or lysozyme (Pressman et al., 1954; Goodman & Baxter). Furthermore, Liu et al., using the passive haemagglutination method to titrate anti-digest sera found that positive results were obtained when tanned cells were used but not when non-tanned cells were used. Tests using purified antigens from tubercle bacilli to inhibit the agglutination of red cells by antisera have provided information about/

about the nature of the antigens adsorbed by tanned and non-tanned cells (Middlebrook & Dubos, 1948; Grabar et al., 1952). It is now generally accepted that protein antigens require tanned cells, whereas polysaccharide antigens will adsorb on to non-tanned cells.

The human source of the digests used in the present work precluded nephrotoxicity tests. However, it was hoped to confirm the specificity of the antibodies and demonstrate the histological distribution of the antigen(s) by tissue-localisation experiments. It is difficult to explain the failure of the antidigest sera to inhibit staining with anti-human-glomerulus serum otherwise than that ^{the} antibody titre was too low. This is also likely to be the reason why the labelled anti-digest sera gave no reaction. The titre of the present sera was not determined after conjugation, but a fall in titre such as that observed by Fabius (see Section V, p. 166) would certainly eliminate any reaction on sections. This difficulty could be overcome by using the indirect method, but unfortunately no labelled anti-rabbit globulin was available when these experiments were performed.

The histological source of the glomerular digests has not been defined with greater precision than/

than that they are derived from glomeruli. However, it seemed justifiable at the outset of these experiments to assume that the digests might be derived from the nephrotoxic antigens. Cole et al. had found that extraction with saline, alkaline and acid solutions, alcohol and diethylene glycol failed to bring the nephrotoxic antigen of rat kidney into solution. Eisen & Pressman (1950) showed that the antigen which absorbs ^{131}I -labelled kidney-localising antibody from rabbit anti-rat-kidney serum was not soluble in saline, acetone or ether. Krakower & Greenspon (1951) demonstrated that the antigen is concentrated in basement membrane, isolated by ultrasonic vibration and optically free of cells; isolated glomerular cells were found to be 50 times less antigenic than basement membrane on the basis of nitrogen content. The results presented in Section II of this thesis appeared to confirm that the basement membrane was the nephrotoxic antigen. Thus the tryptic digests were assumed to be derived from this insoluble basement membrane. Subsequent work which supported this assumption was the observation that the concentration of nephrotoxic antigen in immature glomeruli, where the basement membrane is thin, is less than in mature glomeruli (Krakower & Greenspon, 1954).

However/

However, there is considerable evidence which indicates that this assumption is erroneous. First, the reaction obtained in the present experiments between anti-digest sera and saline extracts of whole kidney (Table XI) suggest that the glomerular digest was derived in part from a saline-soluble source, for the saline extract had been spun free of suspended material. Lippmann et al. (1950) found that at least 12 per cent of the globulin in anti-rat-kidney serum was precipitated by the saline-soluble portion of a kidney suspension. Second, Baxter & Goodman (1956) observed that saline extracts of rat kidney had some effect in absorbing nephrotoxic antibodies from rabbit antisera. Third, Rothenberg et al. (1956) found that the passive haemagglutination titre of anti-rat-kidney serum, using a saline extract of rat kidney as antigen, is closely correlated with the nephrotoxicity of the serum. Fourth, and even more significant, Liu et al. noted a good correlation between the nephrotoxicity of rabbit anti-rat-kidney sera and its cytotoxic effect for rat kidney cells in tissue culture. Finally, Dresner & Schubert (1955) have shown that trypsin causes only slight hydrolysis of renal basement membrane.

It/

It must be concluded, therefore, that tryptic digests of glomeruli represent mainly cellular breakdown products; digests of whole kidney contain an even greater proportion of such material. Furthermore, the relative importance of glomerular basement membrane and cells as the antigens responsible for nephrotoxic nephritis must be regarded as still unproven. Goodman & Baxter have shown that tryptic digests of rat lung, skeletal muscle, heart and liver also contain antigens capable of absorbing nephrotoxic antibodies from anti-rat-kidney serum. It seems likely, therefore, that endothelial cells, which are common to all these organs, rather than epithelial cells peculiar to the glomeruli should be investigated as the source of a nephrotoxic antigen. Until this point has been settled, there is little to be gained from chemical analysis of digests.

SUMMARY

Immunological studies have been conducted on six tryptic digests of human glomeruli and three tryptic digests of whole human kidney.

The digests have been shown to contain haptenic/

haptenic material specific to the glomerulus.

Rabbits were immunised with both types of digest alone, adsorbed on to streptococci and in Freund's adjuvant. The digests themselves were antigenic and no adjuvant effect was noted. The antisera reacted with the digests and also with a saline extract of whole human kidney. Attempts to localise the antigen(s) with fluorescein-labelled antisera were unsuccessful.

The results are discussed in relation to other work on tryptic digests of kidney and on nephrotoxic nephritis.

It is concluded that the antigenicity of the digests is due to protein rather than polysaccharide and that they consist mostly of cellular breakdown products rather than material derived from basement membrane.

THE ROLE OF "AUTO-ANTIBODIES" IN HUMAN GLOMERULONEPHRITIS.

Although the literature contains many articles purporting to describe the occurrence of auto-antibodies to kidney tissue in human glomerulonephritis, the role of such substances is still uncertain. Thus, few workers have established/

established that the reactive substance in the serum is gamma globulin and few have attempted to identify accurately the antigen or hapten in kidney tissue or extracts. Furthermore, some of the techniques used are known to be unreliable. The object of the present study was to obtain information about the presence of a pathogenetic tissue antibody in acute glomerulonephritis. Evidence has been sought of a direct reaction between serum and human kidney or its derivatives and the serological specificity of such reactions have been investigated. Tissue-localising antibodies have been sought by the fluorescent antibody technique. Attempts have been made to elute antibodies from isolated glomeruli and the eluates have been subjected to the same procedures as the sera. The results have been presented at an International Symposium on Immunopathology (Cruickshank, 1958).

METHODS

Sera.

Sera and glomeruli were obtained as indicated in Table XIV. Acute appendicitis and chronic cholecystitis were chosen as non-renal diseases, more or less comparable in their general features with acute and chronic nephritis and unlikely/

TABLE XIV - Material studied.

Diagnosis	Total patients	Serum only	Glomeruli only	Serum and Glomeruli
Acute glomerulonephritis	25	20*	2	3
Anaphylactoid purpura with nephritis	9	9†	-	-
Subacute glomerulonephritis	3	3	-	-
Chronic glomerulonephritis	5	4	-	1
Type II nephritis	6	5/	-	1
Disseminated lupus erythematosus	8	7	-	1
Polyarteritis nodosa	2	2	-	-
Amyloidosis	2	-	2	-
Acute appendicitis	20	20	-	-
Chronic cholecystitis	6	6	-	-

* Two samples from one patient

† Two samples from two patients

/ Four samples from one patient

unlikely to be associated with an immune mechanism. Rabbit anti-human-glomerulus serum (Rabbit 169) was used as a positive control. Details about the cases of acute glomerulonephritis are shown in Table XV. The diagnosis was confirmed histologically in all cases where glomeruli were obtained, in the remaining cases of disseminated lupus erythematosus, by biopsy in one case of anaphylactoid purpura and in all cases of appendicitis and cholecystitis. The source of all sera will be found in the Appendix, pp. 205 - 207.

Sera were separated, clarified when necessary by the method of McFarlane (1942) and stored in the deep-freeze until used. Some of them were fractionated with ammonium sulphate to give euglobulin, pseudoglobulin and albumin fractions. The globulin precipitates were dissolved in phosphate buffer at pH 7.0, dialysed free of $\text{SO}_4^{''}$ and brought to the original volume by the addition of buffer or dialysis against 15 per cent polyvinylpyrrolidone in phosphate buffer. The albumins were dialysed free of $\text{SO}_4^{''}$ and reduced to the original volume by the same method. Several of these fractions were analysed by filter paper electrophoresis.

Samples of those sera which gave positive reactions/

TABLE XV - Cases of acute glomerulonephritis.

Serial number	2	3	7	8	10	11	13	14	20	21	23	27	29	30	31	41	42	48	50	67	70	92	110	111	115
Sex	M	M	M	M	M	M	F	M	M	F	F	F	M	M	M	M	F	F	M	M	M	F	M	M	M
Age	13	51	60	16	5	5	31	48	5	29	10	6	44	4	5	4	7	4	11	6	46	39	1	33	15
Time between onset of disease and collection of serum	2w	6w	2w	1w	3w	?	5d	2w	2w	1w	10d	2w	2w	3w	2w	?	4d	/	?	2d	?	4w	2w	2w	3w

105.

* Serum and glomeruli

† Glomeruli only

/ Two samples obtained: a) in remission, b) 2 weeks after onset of recurrence.

w = weeks; d = days.

reactions were absorbed with a wet preparation of normal human glomeruli in the proportions of 1 mgm/ml. This absorption was repeated a second time if the reaction was unchanged by one absorption.

Eluates.

Glomeruli were isolated by the method used previously, all preparations being kept at 4°C. during the isolation and washing (see Appendix, pp. 186). Elution was performed by methods which have been proved successful in other studies of tissue antibodies, namely, heating at 60°C. (Korngold & Pressman, 1953), N sodium hydroxide at pH 11 (Pressman & Sherman, 1951) and 0.2M citric acid at pH 3 (Talmage et al., 1954). The method of Heidelberger & Kendall (1936), using 15 per cent saline followed by barium salts was also used. Samples of diseased glomeruli varying from 0.1 to 0.5 gm. were used, the same quantity of each preparation being subjected to each of the procedures. A further sample of each preparation was extracted with normal saline at 37°C. for 1 hour as a control. Samples of normal glomeruli were subjected to each of the procedures. Details of the elution procedures are given in the Appendix (pp.199-200 and the source of the glomeruli on pp.205-207).

The eluates were spun to remove insoluble material/

material and the supernates separated. An equal volume of saturated ammonium sulphate was added and the tubes stood overnight at 4°C . They were then spun, the precipitates dissolved in phosphate buffer and dialysed until free of SO_4^{--} . The volume was adjusted to 2 ml. in each tube and any insoluble material spun off. The tubes were then stored in the deep-freeze until used. The globulin concentrations of the eluates were determined by the method of Kingsbury et al. (1926) and from the results obtained the amount of globulin per gm. (wet weight) of glomeruli was calculated.

Antigens.

The "antigens" used were tryptic digests of normal human glomeruli (7,13) or kidney (9,11 & 12) used in the preceding experiments (pp. 81 - 100), a saline extract of whole normal human kidney (Rothenberg et al., 1956) and a suspension of trypsinised normal human glomeruli (Milazzo, 1957). Details of preparation of the antigens are given in the Appendix (pp. 187 & 198)

Serological Procedures.

Sera and eluates were tested for precipitins against the tryptic digests and saline extract by the interfacial ring method and the capillary/

capillary tube method and for complement fixing antibodies against all the antigens by the method used in Section II (pp. 58). The sera were also tested for tissue-localising antibodies by the direct and indirect fluorescent antibody methods. The general principles of these experiments has already been described (Section I). Fresh frozen sections of normal human kidney were treated with the various sera. In the direct method, samples of the patient's sera were labelled with fluorescein isocyanate, the globulin fraction salted out and absorbed twice with acetone-dried guinea-pig liver powder. In the indirect method the procedure of Weller & Coons (1954) was followed, namely, sections were treated with unlabelled patient's serum, washed for 10 minutes in phosphate buffer and then treated with the globulin fraction of

The almost complete absence of cross-reactions between this antiserum and purified fractions of human plasma is seen in Fig.44. Fig.45 shows that a single band of precipitation, with the reaction of identity, occurred when unlabelled and labelled samples were tested against purified gamma globulin.

and inhibition of staining; the remaining sera were tested by all the methods described. Only the indirect fluorescent antibody method was used in/

1

6

2

5

3

4

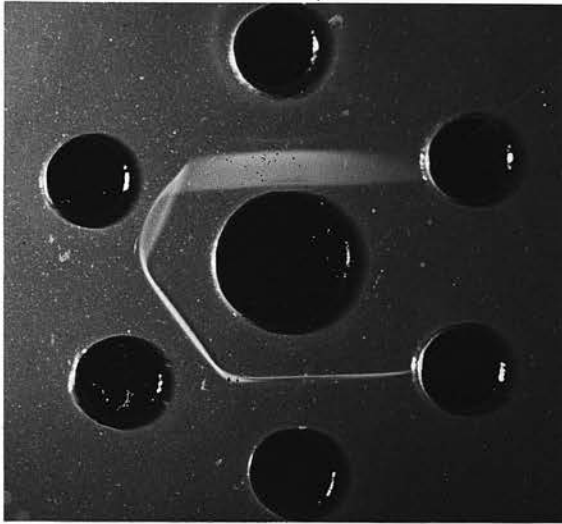


Fig.44. Ouchterlony plate. The centre cup contains anti-human globulin serum. The peripheral cups contain serum (1), albumin (2), α & β globulins (Cohn Fraction IV) (3), γ globulin (4 & 5 - two different samples) and antihaemophilic globulin (6). Note the single band between the antiserum and cups 4 & 5: γ globulin is responsible for the bands between the antiserum and cups 1 & 6.

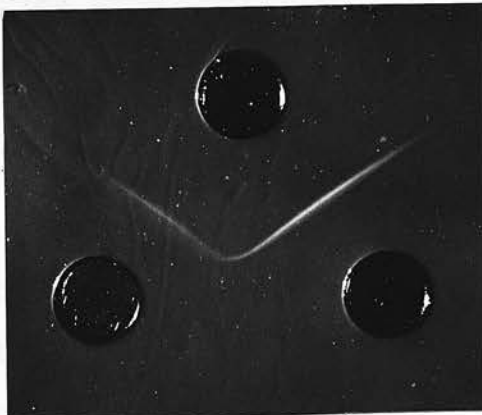


Fig.45. Ouchterlony plate. The upper cup contains human γ globulin, the lower left unlabelled anti-human- γ globulin and the lower right fluorescein labelled anti-human- γ globulin.

in testing the eluates.

RESULTS

The overall results are shown in Table XVI. Only one positive result was obtained in precipitin tests, when one of four samples of serum from a case of Type II nephritis reacted with the tryptic digests. Absorption with glomeruli had no effect upon the reactions. No reaction was obtained between this serum and the other antigens, complement fixation tests with the digests were negative and no tissue localising antibodies were demonstrable. This case is of considerable interest for the nephritis commenced shortly after immunisation with poliomyelitis vaccine; the relationship between these two features will be discussed later (pp. 142). None of the sera or eluates gave positive results in agglutinin tests. No evidence of specific tissue-localising antibodies was obtained with any of the sera or eluates.

Positive complement fixation tests were obtained with 16 sera; 10 others were anticomplementary. The titres obtained with the various antigens are shown in Table XVII. Most of these reactions/

TABLE XVI - Results of tests with sera.

Diagnosis	Precipitin - HKD		All tests	
	Staining - inhibition	Total	Pos.	Neg.
Acute glomerulonephritis	8	-	8	16
Anaphylactoid purpura with nephritis	-	-	-	11
Subacute glomerulonephritis	1	-	1	2
Chronic glomerulonephritis	2	-	-	3
Type II nephritis	-	-	3	10
Disseminated lupus erythematosus	-	-	2	8
Polyarteritis nodosa	-	-	1	2
Acute appendicitis	-	-	2	20
Chronic cholecystitis	-	-	-	6

Figures in brackets indicate anticomplementary sera.

HKD Tryptic digest of kidney.

TABLE XVII - Complement-fixation tests - absorption of sera.

Diagnosis	Serum No.	Complement-fixation test - titre			
		before absorption	after absorption	HKD	KE GS
Acute glomerulonephritis	23	16	-	1	..
	27	-	1	..	a/c
	29	16	-	16	..
	30	16	-	64	..
	31	16	-	16	..
	41	2	-	-	..
	48B	2	-
	70	4	..	a/c	..
Subacute glomerulonephritis	38	-	1
Type II nephritis	24	256	-	1	..
Disseminated lupus erythematosus	43	-	1
	36	-	1	..	1
Polyarteritis nodosa	63	1	1	-	16
	35	2	-	-	..
Acute appendicitis	81	16	-	16	..
	88	1	1	1	..

Results expressed as the reciprocals of the serum dilutions.

a/c Anticomplementary.

HKD Tryptic digest of kidney

KE Saline extract of kidney

GS Trypsinised glomeruli

reactions were obtained with tryptic digests of whole kidney and one such preparation (9) was used for further tests after absorption or fractionation of the sera. Similar reactions were obtained with tryptic digests of isolated glomeruli. The effect of absorbing the reacting sera with normal human glomeruli is also seen in Table XVII. Reduction in titre by more than one tube was seen on two occasions. In the remaining instances, initial titres of 1/1 or 1/2 were abolished or the absorption produced no diminution. Indeed, in two instances increase in titre by one or two tubes occurred, even after a second absorption. Sufficient material for fractionation of five sera was available. The fractions, adjusted to the volume of the sample from which they were derived, were tested against the antigen(s) with which the original serum had reacted, and the results of tests with these fractions are seen in Table XVIII. Insufficient antigen was available to perform all of these tests with serial dilutions of the serum fractions. Positive results were not confined to the euglobulin fractions. Filter-paper electrophoresis indicated that all the albumin fractions and the pseudoglobulin fraction of serum 29 were free of γ -globulin and that the pseudoglobulin fractions of sera 81 and 88 contained approximately one-half and one-fifth of the/

TABLE XVIII - Complement-fixation tests - fractionation of sera.

Diagnosis	Serum No.	Euglobulin	Pseudo- globulin	Albumin
Acute glomerulo- nephritis	27	++ [*]	a/c	+
	29	-	++	-
	31	a/c	a/c	++
Disseminated lupus erythematosus	36	++	a/c	±
	63	-	++	-
Acute appendicitis	81	4	16	-
	88	1	4	-

* Where reciprocal of serum dilution is not given, only the undiluted fraction was used.

the γ -globulin present in the corresponding euglobulin fractions. The fractions of serum 63 were not analysed electrophoretically.

The results of tests with the eluates are shown in Table XIX. No attempt was made to titrate serial dilutions of these preparations. Positive results occurred only in complement fixation tests with the tryptic digests. It should be noted that the eluates from normal and amyloid glomeruli gave similar reactions to those from nephritic glomeruli and that there is no correlation between the globulin concentrations and the results of the complement fixation tests.

DISCUSSION

Witebsky et al. (1957) have recently suggested certain criteria which should be fulfilled in order to prove the role of an auto-antibody in the pathogenesis of a disease. It is pertinent to discuss the present and previous results in glomerulonephritis in relation to these criteria.

'Direct demonstration of free, circulating antibodies that are active at body temperature or of cell-bound antibodies by indirect means.'

A pathogenetic auto-antibody must, by definition, react with the patient's own tissues. Neither the present experiments nor any of those described/

TABLE XIX - Globulin concentration of and complement-fixation tests with eluates

Diagnosis	Specimen no.	Heat		Citric acid		NaOH		Salt + Ba. salts		Control	
		Globulin mgm/ gm 100ml.	CF test	Globulin mgm/ gm 100ml.	CF test	Globulin mgm/ gm 100ml.	CF test	Globulin mgm/ gm 100ml.	CF test	Globulin mgm/ gm 100ml.	CF test
Acute glomerulonephritis	67	<0.1	<1		++		++		+	0.4	2
	92	0.2	6	0.1	2	1.5	37.5	0.7	17	0.8	21
	110	0.4	2		++		++		+	0.6	3
	111	0.8	19	0.1	1.5	1.4	34	1.1	28	0.3	7
	115	0.8	8		++		+		+	0.5	5
Chronic glomerulonephritis	15	0.4	2		++		++		++	0.4	2
Type II nephritis	16	0.3	3		++		+		++	0.4	2
Disseminated lupus erythematosus	68	2.2	11		++		++		++	0.8	4
Amyloid	116	1.1	28	0.7	17	1.5	37.5	1.2	29	0.2	6
	117	2.4	61	2.0	50	7.5	187	1.3	32	0.4	10
Normal control		0	0	0.2	5	2.4	51	0.4	8	0.7	19

Globulin concentrations are expressed as mgm/gm. wet wt. glomeruli and mgm/100 ml. eluate.
Toluates used undiluted in complement fixation tests.

described previously have attempted to show that the serological changes are those of a true auto-antibody. The reagents involved have been serum and the tissues of another human subject. Any antibodies demonstrable in such experiments are really a form of iso-antibody - a homo-antibody, in transplantation terminology. However, the results of studies with other tissue antigens, such as brain, lens, sperms and thyroglobulin suggest that such an antibody would also act as a true auto-antibody. The term "auto-antibody" is used in this thesis with this reservation.

No evidence of any agglutinating or tissue-localising activity has been found in the present study, either in the serum or in eluates. A precipitin reaction was obtained with one of four samples of serum from one case of Type II nephritis, but this was proved non-specific by an absorption test and by inability to get a reaction with that serum in other tests. Several nephritic sera and most eluates gave positive complement fixation tests which were regarded as non-specific for the following reasons. Firstly, absorption with glomeruli had no significant effect upon the reaction except in two instances. Secondly, similar reactions occurred with sera from cases of acute appendicitis/

appendicitis and eluates from normal glomeruli; these results also were unaffected by absorption. Thirdly, reactions occurred at the same or higher titres with serum fractions from which γ -globulin was absent, or in which the concentration was much reduced, as were found with fractions containing normal serum concentrations of γ -globulin. Fourthly, there was no correlation between the protein concentration in the eluates and the intensity of the reactions; indeed, reactions occurred with some preparations which probably contained no protein. It should be emphasised that the highest total protein concentration in eluates from nephritic glomeruli was one five-hundredth of the normal serum level. Fifthly, none of the other tests with sera or eluates which gave these complement fixation reactions was positive. There is thus no concrete evidence that antibodies were responsible for the reactions observed.

These results are supported by those of Spielmann et al. (1954) who obtained complement fixation reactions in controls as well as in nephritics using the original Wassermann technique, but no reactions using 50 per cent haemolysis as the end-point. They concluded that certain proof of auto-antibodies by the complement fixation technique/

technique is not possible. Kay et al. (1941) had previously been unable to demonstrate complement fixing auto-antibodies in post-scarlatinal nephritis. A critical examination of other previous work indicates that the nature of the serum component responsible for complement fixation (Vorlaender, 1952; Wendelberger & Fröhlich, 1955) or passive haemagglutination (Vorlaender, 1955; Vorlaender & Nüssgens, 1957) has not been identified. Gadjusek (1958) has demonstrated a complement fixation reaction, which is a property of the globulin fraction, but which lacks either tissue- or species-specificity. Furthermore, this so-called 'auto-antibody' did not react with antigens prepared from the patient's own tissues (Mackay et al., 1957). Absorption tests to establish the specificity of the reactions do not appear to have been performed by any of these workers. The same criticisms apply to results obtained with the collodion particle technique, which is notoriously unreliable (Morris, 1942; Cavelti, 1947); indeed, some of those who have used it for this problem have themselves commented on this point (Lange et al., 1949; Bialestock, 1951).

'Recognition/

'Recognition of the specific antigen against which this antibody is directed.'

Only one of the antigens used in this work has been identified histologically, namely, the suspension of trypsinised glomeruli. This is cell-free and consists of the basement membrane of the glomerular tufts and Bowman's capsule, with small quantities of tubular basement membrane. Similar preparations, obtained by maceration in dilute alkali, have been studied in detail and accurate information is available about the physical, biochemical and histochemical properties (see Section II, pp. 44 - 45). Milazzo has shown that the antigenicity of glomerular basement membrane is not impaired by tryptic digestion. Only one nephritic serum in the present study reacted with this antigen. The reaction occurred in a complement fixation test, was not affected by absorption with glomeruli and was not confirmed by the other techniques used. Some features of the tryptic digests have been discussed in the preceding Section (pp. 97 - 100). The saline extract of whole kidney is similar to those used by most previous workers in serological tests in nephritis. Its histological and chemical nature is not known, but it must contain serum proteins and soluble materials derived from many/

many cells and interstitial tissues: the basement membrane is not represented as it is insoluble in saline. Glomerular cells have not been present in undegraded form in any of the kidney extracts used in this or previous work in human nephritis. If they can be prepared from human material, the specific mixed agglutination technique of Coombs et al. (1945) might be applied. However, no immunohistochemical evidence of an antibody to glomerular cells could be demonstrated in the present work. Studies with tryptic or saline extracts may be complicated by the fact that they may give reactions such as those described by Kidd & Friedewald (1942a,b) in rabbits and attributed by them to normal tissue antibodies. The antigen responsible for this reaction was present in highest titre in the kidney. There is some evidence that such an antibody occurs in man (Thomas et al., 1943). Fischel (1957) suggests that a false positive Wassermann reaction has also to be considered in assessing complement fixation tests.

The present negative and inconclusive results might be attributable to the techniques used. However, all of these and all of the tissue extracts have been of some value in studies of antisera to kidney or glomeruli produced experimentally in a heterologous species.

Furthermore/

Furthermore, precipitin, complement fixation and tissue localisation studies with the fluorescent antibody method have been successful in demonstrating circulating antibodies in human diseases such as Hashimoto's disease (Roitt et al., 1956; Trotter et al., 1957; White, 1957) and disseminated lupus erythematosus (Holborow et al., 1957; Seligmann, 1958). It is, of course, possible that the tests used in the present work were not sufficiently sensitive and that small quantities of antibody might have been detected by passive haemagglutination. However, Stavitsky (1954) has pointed out that the great sensitivity of haemagglutination reactions may be a drawback with impure antigens. It is also possible that the sera were not obtained at a time when antibodies were present. Sixteen of the sera were collected two weeks or less after the onset of the disease and five of these within one week of onset. If circulating tissue antibodies are responsible for the renal damage they should have been detected in at least some of the samples.

Theoretically, there are good reasons why antibodies to glomerular components are unlikely to be found in the serum, even if they are formed. First, the amount of tissue available for combination with any such antibody is so great that/

that enormous quantities would have to be produced to attain any excess free in the circulation. Pressman & Eisen (1950b) were unable to saturate the anti-kidney antibody-binding sites in the mouse with 15 mgm. of globulin from a rabbit anti-mouse-kidney serum. The close antigenic relationship between the basement membrane of the glomerulus and that elsewhere (see Section II) indicates that antibodies directed against the glomerular basement membrane would be bound to many other organs and tissues. This is probably also true for antibodies against glomerular endothelial cells. There is no evidence of excessive activity in the reticulo-endothelial system or of plasma cell infiltration of the kidney in acute nephritis. Second, it is known that circulating heterologous antibodies to kidney are rapidly bound to the tissues (Sarre and Wirtz, 1939; Pressman et al., 1950; Lippman et al., 1952). Auto-antibodies to kidney in human glomerulonephritis would therefore, be much more likely to be bound to tissue than free in the serum. It is usually assumed that the auto-antigen is located in the glomeruli and is produced by the action of a bacterial product. As the infection which precedes acute glomerulonephritis is frequently a streptococcal tonsillitis, it is surprising that no attempt has been made to demonstrate/

demonstrate such a tissue alteration in the tonsil, where the concentration of bacteria and their products is highest. The tonsil could, furthermore, be the source both of an auto-antigen and its specific auto-antibody. Such an auto-antibody might then cross-react with antigenically related normal tissue components in the glomeruli and so produce nephritis. If auto-antigens can be produced in man by this mechanism a significantly high incidence of glomerulonephritis and rheumatic fever is to be expected in those cases of agammaglobulinaemia where the antibody-producing apparatus recovers.

Whatever the source of a tissue-localising antibody it should eventually be demonstrable in the tissue which is the site of the primary pathological lesion, in this instance the glomeruli. Mellors & Ortega (1956), using the fluorescent antibody technique have demonstrated γ -globulin in the glomeruli in human glomerulonephritis. Their results have been criticised by Vazquez & Dixon (1957) on the grounds that comparative studies of other serum proteins were not made and that there are possible non-immunological explanations for the observations. However, the sections prepared by Mellors & Ortega were washed repeatedly/

repeatedly in phosphate buffer before application of the labelled antiserum, so that any soluble γ -globulin was almost certainly removed. Nevertheless, it is difficult to see how this approach could be used to investigate the role of a tissue antibody. It seemed more likely that elution methods, known to be satisfactory for such antibodies, would provide the answer. The non-specificity of the present results with eluates has already been discussed. The protein concentrations in the eluates have been calculated in terms of the amount eluted per gm. of tissue (Table XIX). There is no significant difference between the controls and the cases of nephritis, so it is unlikely that any tissue-bound antibodies have been eluted. As with the sera, the tissue was obtained within two weeks of onset in four of the five cases studied. However, only one of the autopsies was performed within twelve hours of death, so that post-mortem changes may have affected the results.

'Production of antibodies against the same antigen in experimental animals: appearance of pathological changes in the corresponding tissues of an actively sensitised animal that are basically similar to those in the human disease.'

No attempt has been made in these studies to produce either auto-antibodies or lesions in experimental animals. The model upon which most work of this nature has been based is that of Cavelti/

Cavelti & Cavelti (1945). Many workers have repeated this work and been unable to confirm the results. Bohle et al. (1954) reviewed in detail the literature on this and other forms of experimental nephritis and conducted a comprehensive series of experiments. No serological studies were reported but neither clinical nor pathological evidence of diffuse nephritis was obtained. The authors concluded that no one has reproduced diffuse glomerulonephritis with the Cavelti technique or modifications thereof. It is unfortunate, therefore, that they and other writers have used the term "Cavelti-nephritis". Subsequent work by Sarre & Rother (1954) had similar results. The only attempts to produce true auto-antibodies seem to be those quoted by Grabar (1957), who was unable to demonstrate circulating auto-antibodies in rabbits immunised with one of their own kidneys. Unless pathogenetic auto-antibodies and the antigens responsible can be conclusively identified in human glomerulonephritis there is no place for further animal experiments of this type.

CONCLUSIONS

At the present time, none of the four criteria suggested by Witebsky et al. have been fulfilled in respect of human glomerulonephritis. There are undoubtedly features about the disease which/

which suggest that it may be initiated by an immune mechanism but there is no unequivocal evidence that auto-immunisation to kidney is responsible. The role of auto-immunisation in the later stages of the disease is not yet established. Fischel, in reviewing this question, has suggested that the nephrotic syndrome and chronic nephritis may be interpreted as non-allergic sequelae of an initial renal injury. The present work indicates that immunological studies in such patients must be pursued with caution and rigorously controlled.

SUMMARY

Sera from cases of acute glomerulonephritis and other renal diseases have been examined for precipitating, agglutinating or complement fixing antibodies against tryptic digests of normal human glomeruli, cell-free glomerular basement membrane and a saline extract of kidney. No conclusive evidence has been found for the presence of circulating tissue antibodies. Such reactions as were observed were shown to be non-specific.

Eluates prepared from nephritic glomeruli showed no evidence of tissue-bound antibodies but gave similar non-specific reactions.

None/

None of the sera or eluates contained tissue-localising antibodies detectable by the fluorescent antibody method.

The results are discussed in relation to recent work on experimental nephritis and on serological reactions in human nephritis. There is no unequivocal evidence that auto-immunisation to kidney is responsible for the disease.

THE ROLE OF "AUTO-ANTIBODIES" IN ANAPHYLACTOID PURPURA

The aetiology of anaphylactoid purpura is unknown, though it is generally believed to be a manifestation of hypersensitivity. Gairdner (1948) emphasised the importance of streptococcal infection preceding the onset or provoking relapses or exacerbations. Lewis (1956) observed that an upper respiratory tract infection preceded the onset in 73 out of 116 cases; throat swabs were taken in 93 cases and haemolytic streptococci were isolated from 35 of these. Stefanini & Mednicoff (1954) claimed that the serum of patients with anaphylactoid purpura gave a precipitin reaction with extracts of blood vessels.

It seemed desirable, therefore, to determine whether auto-immunisation to vascular tissue, in the manner postulated for acute glomerulonephritis/

glomerulonephritis, occurred in anaphylactoid purpura. The investigation followed much the same lines as that on acute glomerulonephritis.

METHODS

Sera were obtained from 16 patients suffering from the disease, 9 of whom had evidence of renal involvement. Two samples of serum were obtained from each of two patients in the nephritic group. Sera were separated and stored in the deep-freeze until used. Biopsies, taken to include a lesion and adjacent uninvolved skin, were obtained from two of the patients without renal involvement. The sex, age and time between the onset of the disease and collection of serum are shown in Table XX; the source of all sera is given in the Appendix (pp. 205-207).

The "antigens" included all those used in the study of glomerulonephritis, three prepared from normal mesenteric arteries by extraction with normal saline, 5M potassium chloride and trypsin respectively and cell free reticulin prepared from mesenteric arteries by tryptic digestion (as for glomerular basement membrane in the previous study). For details of the preparation of these extracts see Appendix, (pp./

TABLE XX - Cases of anaphylactoid purpura.

No renal involvement								Renal involvement present								
Serial number	39	44	49	56	57	64*	65*	17	40	46	52	53	54	55	59	60
Sex	M	F	M	F	M	F	F	M	F	M	F	M	F	F	M	M
Age	4	7	2	7	4	?	5	?	8	5	7	3	6	10	9	9
Time between onset of disease and collection of serum	5w	2w	2w	9d	1w	?	6d	?	3m	1w	2d [†]	6d	2w	3w [⊗]	2w	4w

* biopsies also obtained
† two sera during separate attacks
⊗ two sera during one attack

d = days
w = weeks
m = months.

(pp. 198 - 199).

The serological procedures were those used in the study on glomerulonephritis. The blood vessel extracts were used only for inter-facial precipitin tests; the arterial reticulin only for agglutination tests. Tests for tissue-localising antibodies were performed on sections of normal skin and kidney and on the two biopsies.

RESULTS

The results are shown in Table XXI. Sixteen sera were negative in all tests, though three of these were anticomplementary. The two positive results occurred in complement fixation tests with a tryptic digest of kidney. The tests were repeated after the sera had been absorbed with glomeruli (see p. 104). The initial titres of 1/1 and 1/2, became 1/4 and negative. None of the other tests with these two sera was positive.

DISCUSSION

The points made in the preceding discussion concerning the non-specificity of the complement fixation tests are again applicable, so that no evidence of antibodies to vascular tissue/

TABLE XXI - Results in anaphylactoid purpura.

	Total sera	Positive	Negative
Cases without nephritis	7	2	5 (1)
Cases with nephritis	11	-	11 (2)

Figures in brackets indicate anticomplementary sera.

tissue has been found. It must be admitted, however, that the blood vessel preparations were not obtained from vessels of the type primarily involved in anaphylactoid purpura, namely capillaries, arterioles and venules (Gairdner). Theoretically, endothelial cells and reticulin throughout the vascular tree should be similar and there is considerable evidence that they are closely related antigenically. Thus, Clark & Jacobs (1950) found that the injection of dogs with rabbit antisera to dog aortic endothelium resulted in widespread haemorrhagic purpura. Similar results following injection of guinea-pigs with rabbit antisera to guinea-pig aortic endothelium are mentioned by Bernard et al. (1955) and Strehler (1951) produced nephrotoxic nephritis in guinea-pigs by injecting rabbit anti-guinea-pig-aorta serum. ^{131}I -labelled rabbit antisera to rat liver blood vessels (Pressman et al., 1951) and rat aorta (Pressman et al., 1952) both localised in liver, kidney and lungs. The work described in Section I of this thesis provides further evidence of the close antigenic relationship between reticulin in arteries and capillaries. Indeed, the only evidence to the contrary is that of Scott (1957), who found that arterial and capillary reticulin were antigenically different. However, his results are not clear-cut (see Table II, p. 46) and/

and have not yet been confirmed.

The absence of undegraded endothelial cells from the "antigens" used must again be noted. It would appear that they were the major antigen in the experiments of Clark & Jacobs and of Bernard et al. Furthermore, purpura is not a feature of nephrotoxic nephritis. If tissue antibodies are concerned in anaphylactoid purpura it seems more likely that they will be directed against the endothelium than the basement membrane of the capillaries.

The argument developed in the preceding discussion concerning the relative importance of tissue-bound and circulating antibodies is equally applicable to the disease now under discussion. The possibility of obtaining tissue from which antibodies could be eluted is remote, but the opportunity should not be neglected if it arises. However, it seems unlikely that auto-immunisation to vascular tissue is responsible for the disease.

SUMMARY

Sera from cases of anaphylactoid purpura have been examined for precipitating, agglutinating or complement fixing antibodies against tryptic digests of normal human glomeruli and artery, cell/

cell-free glomerular basement membrane and arterial reticulin and saline extracts of kidney and artery. No conclusive evidence has been found for the presence of circulating tissue antibodies. Such reactions as were observed were non-specific.

None of the sera contained tissue-localising antibodies detectable by the fluorescent antibody method.

It is concluded that auto-immunisation to vascular tissue is unlikely to be responsible for the disease.

NEPHROTOXICITY STUDIES IN RELATION TO POLIOMYELITIS
VACCINATION.

The British poliomyelitis vaccine, like those produced elsewhere, is grown in tissue cultures of rhesus monkey kidney. In September, 1954 I was asked by the Medical Research Council Subcommittee on Poliomyelitis Vaccine 1) to investigate the possibility that the vaccines might contain antigenic material capable of stimulating the production in man of antibodies which might combine with human kidney and cause glomerulonephritis, and 2) to investigate the immunological relationship between monkey kidney and human kidney. The results of these experiments were submitted in Memoranda to the Subcommittee in May and December, 1955.

Antigenicity of the Vaccine.

The material used in these experiments has been supplied by Messrs. Glaxo Laboratories Ltd. and I am indebted to Dr. W. Wood of those Laboratories for the following information. Before addition of the seed virus, monkey kidney tissue is incubated with the synthetic protein-free culture medium Connaught No. 199 (Morgan et al., 1950) by a method similar to that of Farrell et al. (1953). No animal serum or extraneous protein other than that derived/

derived from the monkey kidney tissue is added. The fluid removed from such cultures contains approximately 20-30 mgm./100 ml. total nitrogen, 12 mgm./100 ml. amino nitrogen and less than 1 mgm./ml. protein nitrogen. Since these values are of the same order as those in the final vaccine, it was decided to use the culture fluid in the present experiments. Before use the fluid was clarified by passing through sintered glass filters of graded porosity according to the usual technique followed in vaccine production. The pore size of the final filter is 0.7 - 3.0 microns. Thus, the spent culture fluid used differs from the final vaccine only in the absence of the virus.

Two experiments were performed:

- 1) production of generalised anaphylaxis in guinea-pigs. Six animals (14-19) received sensitising doses of 2 ml. intraperitoneally and shocking doses of 10 ml. intravenously 10 days later. No reaction occurred.
- 2) production of antibodies in rabbits. Two animals (156 & 157) received a total of 25 ml. each in 12 divided intravenous doses at two-day intervals. They were bled 18 days after the last dose. The sera were tested by the precipitin ring reaction using serial dilutions of both sera and culture fluid. No reaction occurred. Each serum was then tested in 3 guinea-pigs (20-25) for its ability to produce passive cutaneous anaphylaxis by the method of Ovary (1952). No reaction occurred. Both rabbits subsequently produced antibodies to human gamma globulin.

Details of the injection schedules are given in the Appendix (pp. 196 & 198)

Antigenic relationship between monkey kidney and human kidney.

Two rabbits (144 & 145) were immunised with a suspension of whole monkey kidney, by a procedure similar to that used previously (Section II; protocols in Appendix, p. 196). Antibodies to monkey serum and red cells were removed by absorption and the sera conjugated with fluorescein. Sections of monkey kidney treated with one of the labelled sera and examined in the fluorescence microscope showed staining in the same sites as had previously been demonstrated with antisera to rat kidney (Hill & Cruickshank, 1953), namely the basement membrane of glomeruli and tubules, reticulin in vessels and tubular epithelium. The specificity of this staining was established by inhibition and absorption tests as described previously (Section II, pp. 8 - 11). The other labelled serum failed to react.

The application of the active labelled serum to sections of human kidney produced precisely the same pattern of staining, also due to specific antigen-antibody union.

Antihuman glomerulus sera used in the experiments described in Section I and anti-human-kidney sera prepared subsequently (Section III, p. 84) produced identical staining in monkey/

monkey kidney to that seen in human kidney.

Antibodies to kidney in the sera of vaccinated human subjects.

In view of the completely negative results following immunisation of guinea-pigs and rabbits no systematic examination of the sera of vaccinated human subjects has been undertaken. This decision was endorsed by the Medical Research Council Subcommittee on Poliomyelitis Vaccines. However, one patient encountered in the study of auto-antibodies in nephritis is of interest for the attack of nephritis commenced shortly after the second injection of poliomyelitis vaccine.

Abstract of Case Notes:

Christopher M. aet 2½.

3.7.56. Admitted Belvidere Infectious Diseases Hospital.
No past illnesses. No personal or family history of allergy or sensitivity; no adverse reaction to diphtheria immunisation at 9 months.

Present Illness:

25.5.56. 1st poliomyelitis vaccination - Glaxo Batch 5A.
26.5.56. Developed generalised itchy rash, more profuse over arms, and leg of inoculated side. Red spots with small blister on top. Rash lasted for a week.
Diagnosis: papular urticaria.
22.6.56. 2nd poliomyelitis vaccination - Glaxo Batch 6E.
26.6.56. Eyes puffy, becoming more severe over next week with oedema developing in abdomen, penis and legs. Urine dark and smoky.

Condition on Admission:

Remains/

Remains of rash on legs. Oedema of abdomen, penis, legs and feet. Throat a little red.

Laboratory Investigations:

- 5.7.56. Albuminuria +++ (14 gm./litre); granular casts; blood ++. Antistreptolysin O 125 units./ml.
 6.7.56. Serum proteins 5.8 gm./100 ml.; albumin 2.0 gm./100 ml.; globulin 3.8 gm./100 ml.
 Blood urea nitrogen 32 mgm./100 ml.
 9.7.56. Throat swab - haemolytic streptococci.
 14.7.56. Transferred to Royal Hospital for Sick Children, Yorkhill.
 Diagnosis: Type II nephritis.
 16.7.56. Prednisolone commenced.
 21.7.56. Serum proteins (after diuresis). Total 4.98 gm./100 ml. α -globulin 1.31, β -globulin 1.02, γ -globulin 0.81, albumin 1.84.
 30.8.56. Discharged.
 21.3.57 - 9.5.57) Readmitted with relapse.
 3.10.57 - 9.11.57) Responded to prednisolone on both occasions.

Samples of serum obtained on 5.7.56, 8.8.56 and 29.8.56 gave no reactions in precipitin, agglutination and complement fixation tests with the antigens described previously and no evidence of tissue-localising antibodies. A sample obtained on 31.7.56 reacted in the precipitin ring test with a tryptic digest of whole kidney to a dilution of 1/4; following absorption of the serum with glomeruli, only neat serum gave a reaction. A complement fixation test was performed with the same reagents. The undiluted serum was anticomplementary but satisfactory for testing at a/

a 1/4 dilution; the result was negative. No reaction was obtained in a precipitin or complement fixation test with the saline extract of kidney or in an agglutination or complement fixation test with cell-free glomerular basement membrane. No tissue localising antibodies were detected.

DISCUSSION

Although it is commonly believed that the antigen responsible for nephrotoxic nephritis is located in the glomerular basement membrane, several observations suggest that the glomerular cells may play some part in the process (see pp. 99 - 100). The method of preparation of the monkey kidney cells for tissue culture probably removes the greater part, if not all, of the basement membrane. The subsequent treatment of the culture after inoculation with the virus is such that any small portions of basement membrane originally present are excluded from the vaccine. The cells used in the tissue culture are derived mainly from renal tubules. Cortical tubular epithelium is related antigenically to glomerular epithelial cells (Hill & Cruickshank; Goodman et al.; 1955) and may therefore be nephrotoxic. However, whole cells are not present in the vaccine, though soluble products and insoluble particles resulting from/

from metabolism of the cells or the cytopathogenic effect of the virus will be present (Robbins et al., 1950; Melnick, 1955). Mayer et al. (1957) have shown that infected monkey kidney tissue culture fluid contains monkey kidney antigen; with cultures of trypsinised cells this antigen was removed by ultracentrifugation. The close immunological relationship between monkey kidney and human kidney indicated by the cross-reaction obtained with the rabbit antisera suggest that immunisation of either species with kidney tissue of the other would be difficult, probably requiring large doses of antigen and a strong adjuvant. This information is interesting, but of secondary importance to the antigenicity of the vaccine. The present experiments indicate that filtered spent culture fluid contains no antigenic material resulting from cellular metabolism. No attempt has been made to test the final vaccine for such antigens. Although this may seem theoretically desirable, it was not considered necessary by the Medical Research Council Subcommittee on Polio-myelitis Vaccines. It should be noted, in this respect, that the final vaccine and the spent culture fluid contain the same quantity of protein nitrogen and furthermore that no adjuvant activity has been demonstrated in any known virus.

The decision against examining the sera
of/

of vaccinated human subjects is endorsed by the work of Neva & Salk (1955) who found no urinary abnormality or changes in serum chemistry in patients inoculated repeatedly with poliomyelitis vaccine: there is no other known example of renal disease which might be causally related to vaccination than the one cited here (Macrae, 1958). On these grounds alone it may be questioned whether the vaccination was responsible for the nephritis. Furthermore, there is no confirmatory evidence that the single serological reaction obtained was due to antibodies to kidney. The occurrence of urticaria immediately after the first injection must not be overlooked, for allergic reactions of a minor nature have been reported after vaccination (World Health Organisation, 1956). However, the urticaria did not recur after the second injection. In considering the aetiology of the nephritis it must not be forgotten that haemolytic streptococci were isolated from a throat swab shortly after admission to hospital. A streptococcal infection prior to the onset of the illness was not established, but it cannot be excluded. The precise aetiology of the nephritis cannot be determined; the relationship to the vaccination may well be fortuitous.

SUMMARY/

SUMMARY

Fluid from monkey kidney tissue cultures was investigated for the presence of antigenic material. Attempts to produce anaphylaxis in guinea-pigs and circulating antibodies in rabbits were unsuccessful.

Fluorescein-labelled rabbit antisera to human kidney and monkey kidney gave identical staining reactions in the two species. It is unlikely that monkey kidney could stimulate anti-body production in man.

The occurrence of nephritis shortly after vaccination is reported in a $2\frac{1}{2}$ year old boy. The presence of anti-kidney antibodies was not established. The relationship of the nephritis to the vaccination is discussed.

SECTION IV

IMMUNOLOGICAL STUDIES OF HUMAN ANTERIOR

PITUITARY HORMONES

IMMUNOLOGICAL STUDIES OF HUMAN ANTERIORPITUITARY HORMONES

The precise cellular localisation of the anterior pituitary hormones is not possible with any of the usual histological or histochemical techniques. Nevertheless, it is generally supposed that growth hormone and prolactin are secreted by the acidophils. The association of gigantism and acromegaly with acidophil adenoma of the pituitary is fairly sure proof that growth hormone is secreted by the acidophils in man. Halmi (1950) used a modified aldehyde-fuchsin method and demonstrated two types of basophil in the rat. Pearse (1952), used a periodic acid-Schiff technique on human tissue and showed that no carbohydrate-containing substance was demonstrable in the acidophils and concluded that follicle-stimulating hormone, luteinising hormone and thyrotrophic hormone (TSH), which are generally believed to be glycoproteins, must be secreted by the basophils. Purves and Griesbach (1951, 1954) claimed that three types of basophil could be differentiated in the rat by the periodic acid-Schiff technique and that these are the sites of production of the three glycoprotein hormones. Similar results have been obtained by electron microscopy in the rat (Farquhar and Rinehart, 1954a, b). Barnett, Ladman, McAllaster and Siperstein (1956) used the differential solubility of proteins, various staining procedures and bio-assay methods in an extensive study of the glycoprotein hormones. Their results suggest that the two gonadotrophins may be secreted by the same cell. Purves and Griesbach (1955) were also able to correlate the glycoprotein content of the cells with the hormone content of the gland as measured by bio-assay. The changes described by Russfield (1955) in the basophils in diseases of the human thyroid suggest that TSH is secreted by these cells in man. There is also evidence that adrenocorticotrophic hormone (ACTH) is secreted by the basophils in man. This view is supported by the changes demonstrated in these cells in Cushing's syndrome by Crooke (1935), in Addison's disease by Crooke and Russell (1935), after ACTH^{therapy} by Golden et al. (1950-), after cortisone therapy by Laqueur (1950) and as a result of stress by Currie and Symington (1955a, b). All these/

these findings are only presumptive, for they did not involve the specific staining of any of the hormones.

Marshall (1951), in the first paper to describe the application of the fluorescent antibody technique to the localisation of a tissue component, showed^{that} a labelled antiserum to hog ACTH reacted with basophils in the hog pituitary. He concluded that these cells secrete the hormone. This work has been widely accepted but has not yet been confirmed. In view of this, and of the successful results obtained by Hill and Cruickshank (1953) with kidney antigens, it was decided to attempt to develop specific staining methods for human ACTH, TSH, growth hormone and gonadotrophins, using the fluorescent antibody technique. This section of the thesis describes experiments designed to prepare specific antisera to these hormones, serological studies of those sera and the results obtained with early localisation experiments. The work has been reported by Cruickshank and Currie (1958).

METHODS

Antigens

The hormone preparations used to produce the antisera and in the serological tests with those sera have been extracted from human pituitaries (pituitary extracts) or from human urine (urinary extracts), because other workers have shown that some anterior pituitary hormones are species-specific (Chow, 1942; Van Dyke et al., 1950; Marshall, 1951). The preparations used were:

a) pituitary extracts:

ACTH (Hewett et al., 1954)

TSH (Currie, Cruickshank, Dekanski and Skinner, 1956)

growth hormone, extracted by Mr. L.G. Skinner of Organon Laboratories and tested for activity by Prof. F.G. Young

b) urinary extracts:

post-menopausal gonadotrophin (HMG-20A, Organon)

TSH (Currie, Cruickshank, Dekanski, Hewett and McGirr, 1956).

For ease in reference, the TSH prepared from the pituitary will be denoted 'pituitary TSH' and that prepared from urine 'urinary TSH'. The biological activity of the various preparations is shown in Table XXII. Although most of them have been shown to contain more than one biologically active substance, they will be/

be referred to by the name of the major hormonal component. The use of the term 'antigen' for such a preparation does not imply that there is only one antigenic component.

Several other preparations were used in the serological tests: hog ACTH (Organon) containing 30.9 I.U. per mgm.; ox ACTH (Armour) containing 2.5 I.U. per mgm.; ox growth hormone (Armour) having 75 - 100 per cent activity of the Armour standard preparation; human chorionic gonadotrophin (Pregnyl, Organon) containing 2060 I.U. per mgm.; a kaolin-acetone extract from normal human male urine containing 0.1 units of HMG-20A per mgm. (Loraine and Brown, 1954; Loraine, 1956); purified pregnant mare serum gonadotrophin (Gestyl, Organon) containing 4000 I.U. per mgm; and normal human serum.

Immunisation Procedure

Adult, cross-bred rabbits, kept in individual cages on a mixed diet, were used to produce the antisera. Subtotal adrenalectomy, thyroidectomy or castration were performed in those animals immunised with ACTH, the thyrotrophic hormones or gonadotrophin respectively. These operations were performed in order to prevent rapid utilisation of the injected hormone by its target organ. Details of the operative procedures are given in the Appendix (pp. 190 - 191). Immunisation was commenced two weeks after the various operations.

The hormones were injected as alum-precipitated suspensions in divided doses, either intramuscularly, or intraperitoneally and subcutaneously (Marshall, 1954a); growth hormone was also injected along with Freund's adjuvants. The animals were bled at intervals and the injections continued until the undiluted sera reacted with the homologous antigen diluted to at least 1/1000 parts in saline. In some instances, the final course of injections was of a solution of the hormone in saline, given intramuscularly and/or intravenously. Two rabbits were used for each of these schedules, except where stated to the contrary. Details of the immunisation schedules are given in the Appendix (pp. 194 - 197). When satisfactory titres had been obtained, the animals were bled and the sera to each hormone pooled.

Serological Procedures

Initially, undiluted sera were tested for precipitins against/

Table XXII

Biological activity of extracts

Extract	Biological activity			
	ACTH*	TSHP	SH	HMG
	(IU/mgm)	(H-LU/mgm)†	(gm/mgm)	(U/mgm)
ACTH	2.0	0.05
TSHP	0.015	1.0
SH	0.025	0.1-0.2	111	..
HMG	..	nil	..	1.0
TSHU	0.2	0.25	..	0.8‡

Abbreviations used in this and other tables are:

ACTH - adreocorticotrophic hormone

TSHP - thyrotrophic hormone extracted from pituitary

SH - growth hormone

HMG - post-menopausal gonadotrophin

CG - chorionic gonadotrophin

TSHU - thyrotrophic hormone extracted from urine

MUE - extract of male urine

NRS - normal rabbit serum

* thymus-involution method of Bruce et al. (1952)

† 1 Heyl-laqueur unit is approximately equal to
0.1 International Unit of TSH

‡ ovarian weight method

against the hormones dissolved in saline. Stock solutions of one part of antigen to 1000 parts of saline (w/v) were made up. The pituitary extracts were much less soluble than the urinary extracts; considerable proportions of all of the former failed to dissolve, whereas no such difficulty was encountered with the latter. For use in the tests, the stock solutions were diluted with saline in four-fold steps to $1/256$, giving a final dilution of the hormone of $1/256,000$ (w/v). Precipitin tests were performed by the interfacial ring technique, antigen being layered upon serum. Subsequent tests were performed with serial four-fold dilutions of the antisera in saline against a constant dilution of the various antigens. All of these were used at a dilution of $1/1000$, except normal human serum, which was used undiluted.

Absorption-precipitin tests were performed by adding various antigens to the undiluted sera in the proportion of 1 mg. per ml., allowing the mixture to stand for several hours at room temperature, spinning and then titrating four-fold dilutions of the absorbed sera against a $1/1000$ dilution of the various antigens. Usually a single absorption gave a clear-cut result; occasionally the procedure had to be repeated once.

Agar-gel diffusion precipitin tests were performed by a modified Ouchterlony technique, using 2 per cent Difco agar containing 0.85 per cent saline and $1/10,000$ thiomersalate. The plates were poured in a single layer and the cups cut by hand with cork-borers. Undiluted antiserum was placed in the central cup and $1/1000$ dilutions of antigens in the peripheral cups. Duplicate plates were poured; one plate of each system was incubated at 37°C , the other at room temperature. The cups were refilled with saline when required. When the plates were satisfactorily developed they were photographed, using transmitted light from a Pointolite source and a plate camera.

The antiserum to ACTH (anti-ACTH) was tested for hormone-specific antibodies by its ability to precipitate the hormone from a solution of the extract used to produce it. 15 mg. portions of the extract were added to 20 ml. of the antiserum and to 20 ml. of normal rabbit serum. The mixtures were allowed to stand for several hours at room temperature and then overnight at 4°C . They were then spun, the precipitates discarded/

discarded and the supernatant fluids freeze-dried. Each of the resulting powders was then tested by Dr. J.B. Dekanski of Organon Laboratories for ACTH activity by the thymus-involution method and by the in vitro method of Saffran and Schally (1955). The original extract was retested at the same time by the thymus-involution method.

Localisation Experiments

Globulin fractions of the antisera were labelled with fluorescein isocyanate (see Appendix, pp. 201-202). Sections of fresh-frozen tissues were prepared by the method already described and by the Altmann-Gersh freeze-drying method, followed by fixation in cold methanol (see Appendix, p. 202). All sections were treated with labelled antisera by a modification of the method of Coons and Kaplan (see Appendix, p. 203) and examined in the fluorescence microscope. Tests for the specificity of staining were performed according to the abbreviated method discussed on p. 11; in addition portions of the labelled antisera were absorbed with the homologous antigen before use. The adjacent section to that treated with the fluorescent antiserum or, in some instances, the same section was then fixed in 10 per cent formol-calcium and stained by a modification of the trichrome-periodic acid-Schiff technique of Pearse (1949). Adequate staining of both fresh-frozen and freeze-dried material has been obtained.

RESULTS

Precipitin Tests

The initial precipitin tests were performed with undiluted sera and serial dilutions of antigens, because labelled undiluted serum, or its globulin fraction, is used in the fluorescent antibody technique to detect unknown amounts of antigen in the tissues. It was desirable, therefore, to know the quantity of antigen which could be detected by the various antisera. The final titres obtained with the various antisera against their homologous antigens were:

anti-ACTH: 1/4000 (1 rabbit), 1/16,000 (4 rabbits)
and 1/64,000 (1 rabbit)

anti-pituitary TSH: 1/4000 (1 rabbit)

anti-growth hormone: 1/4000 (2 rabbits)

anti-gonadotrophin: 1/16,000 (1 rabbit) and
1/64,000/

1/64,000 (1 rabbit)
 anti-urinary TSH: 1/4000 (1 rabbit) and
 1/16,000 (1 rabbit).

The reaction between ACTH and anti-ACTH resulted in the loss of 58 - 67 per cent of the activity of the extract (in vitro method) and 75 per cent of the activity of the extract (thymus-involution method).

Serial dilutions of the antisera were titrated for cross-reactions with heterologous antigens, with the results shown in Table XXIII. It will be noted that the antisera to each of the pituitary extracts reacted with all of them, but not with any of the urinary extracts, and vice versa. In particular, the antisera to the two thyrotrophic hormones reacted with their homologous antigens but not with the extract of similar biological activity prepared from the other source.

Anti-ACTH gave no reaction with hog or ox ACTH. None of the antisera reacted with ox growth hormone. Anti-gonadotrophin and anti-urinary TSH reacted with Pregnyl and with the kaolin-acetone extract from normal male urine. Anti-gonadotrophin gave no reaction with pregnant mare serum gonadotrophin. None of the antisera reacted with normal human serum.

Absorption-precipitin Tests

Portions of the antisera to the pituitary extracts were absorbed with, and then titrated against, each of these extracts. The results are shown in Table XXIV. The only reaction remaining after two absorptions was that between anti-growth hormone absorbed with ACTH, and pituitary TSH. No specific antibodies were detected.

Portions of the antisera to the urinary extracts were absorbed with, and then titrated against, each of these extracts. Similar absorptions were performed with Pregnyl and the extract of male urine. The results are shown in Table XXV. No reaction remained. In view of the results in Table XXIII, no attempt was made to perform absorption-precipitin tests in which antisera to pituitary extracts were absorbed with urinary extracts, or vice versa.

Absorption of each of the antisera with 100 mgm. of acetone-dried human liver powder per ml. did not alter the titres with the homologous antigens

Gel-diffusion/

Table XXIIICross-reactions with various antisera

Antiserum to	Antigen						
	ACTH	TSHP	SH	HMG	CG	TSHU	MUE
ACTH	256	4	4	-	-	-	-
TSHP	1	4	1	-	-	-	-
SH	4	16	16	-	-	-	-
HMG	-	-	-	4	4	4	4
TSHU	-	-	-	16	16	16	4
NRS	-	-	-	-	-	-	-

Figures given are the reciprocals of the highest dilution of antiserum which reacted with the antigens, all of which were used at a constant dilution of 1/1000.

Table XXIV

Absorption-precipitin reactions with antisera
to ACTH, to growth hormone and to
pituitary thyrotrophin

Antiserum to	Absorbed with	Antigen		
		ACTH	TSHP	SH
ACTH	-	256	4	4
	ACTH	-	-	-
	TSHP	-	-	-
	SH	-	-	-
SH	-	4	16	16
	ACTH *	-	4	-
	TSHP *	-	-	-
	SH *	-	-	-
TSHP	-	1	4	1
	ACTH	-	-	-
	TSHP	-	-	-
	SH	-	-	-

Results expressed as previously. Antigen
dilutions 1/1000.

* two absorptions.

Table XXV

Absorption-precipitin reactions with antisera
to urinary gonadotrophin and to
urinary thyrotrophin

Antiserum to	Absorbed with	Antigen			
		HMG	CG	TSHU	MUE
HMG	-	4	4	4	4
TSHU	-	16	16	16	4
Both sera	(HMG	-	-	-	-
	(CG	-	-	-	-
	(TSHU	-	-	-	-
	(MUE	-	-	-	-

Results expressed as previously. Antigen
dilutions 1/1000.

Gel-diffusion Tests

Antisera to each of the pituitary extracts were put up against all of these extracts. The result with anti-ACTH is seen in Figure 46. Three lines developed between the antiserum and the homologous antigen, two lines between the antiserum and growth hormone and one line between the antiserum and pituitary TSH. The last-named line fused with one of the other lines between each of the other antigens and the antiserum. This 'reaction of identity' (Ouchterlony, 1953) indicates the presence of an antigen in common to the three preparations. When anti-growth hormone was similarly tested, one line developed between the antiserum and the homologous antigen and two lines between the antiserum and the other two antigens. Fusion of lines was again seen. Only one line developed between anti-pituitary TSH and each of the antigens; fusion was again present. When the absorbed antisera were put up, the only precipitate which developed was between anti-growth hormone absorbed with ACTH and pituitary TSH (cf. Table XXIV).

Antisera to each of the urinary extracts were put up against all of these extracts, including Pregnyl and the extract of normal male urine. Once again, lines which fused developed between the central and peripheral cups (Figure 47). Second lines developed between anti-gonadotrophin and both Pregnyl and the extract of male urine; these lines also fused. Three lines developed between anti-urinary TSH and post-menopausal gonadotrophin. In all other instances only one line developed. There is thus at least one antigen shared by the various urinary extracts. When the absorbed antisera were put up no precipitate developed (cf. Table XXV).

Localisation Experiments

The anti-ACTH and anti-gonadotrophin were labelled and applied to sections before the extent of the cross-reactions was known. Anti-ACTH reacted with granules in the cytoplasm of some cells, identified by the trichrome-periodic acid-Schiff method as basophils, in sections from several pituitaries. However, it was not possible to inhibit this reaction by applying unlabelled antiserum before the labelled sample, nor was it prevented by absorbing the labelled sample with the homologous antigen before use. The staining must, therefore, be/

Cup 1

Cup 3

Cup 2

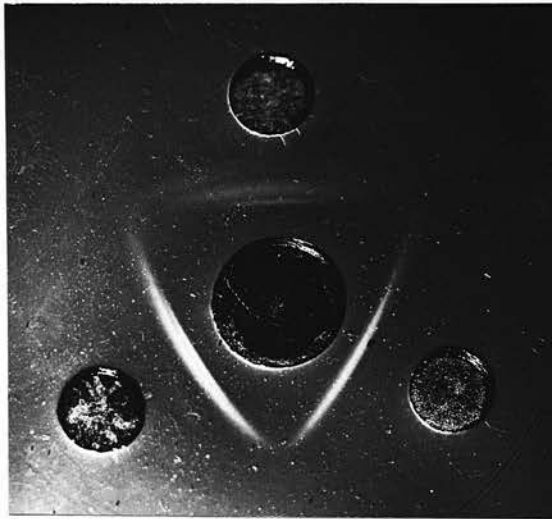


Figure 46. Gel-diffusion reaction between anti-ACTH and ACTH (cup 1), pituitary TSH (cup 2) and growth hormone (cup 3).

Cup 1

Cup 2

Cup 4

Cup 3

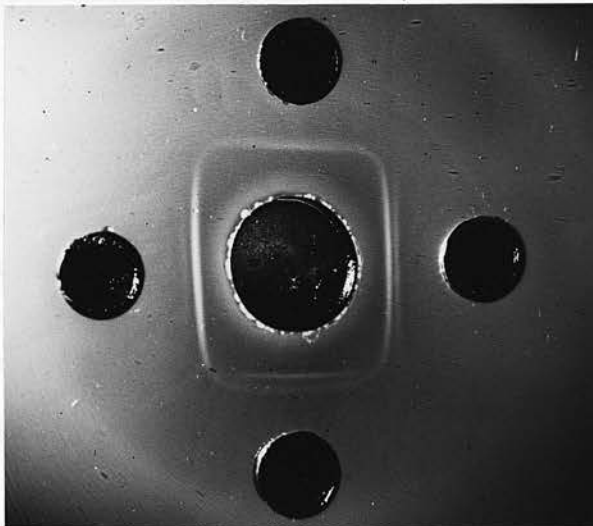


Figure 47. Gel-diffusion reaction between anti-HMG and HMG (cup 1), Pregnyl (cup 2), urinary TSH (cup 3) and extract of male urine (cup 4).

be regarded as non-specific. The pattern of staining with the labelled anti-gonadotrophin was similar, though not necessarily involving the same cells as those reacting with anti-ACTH. Again, it was not possible to demonstrate that the staining was specific. When the same sera ^{which had been stored at -20°C} were used some months later no reaction was obtained with anti-ACTH, whereas anti-gonadotrophin still reacted. The pattern of staining obtained with anti-urinary TSH was again similar and non-specific: anti-pituitary TSH was not labelled. Labelled anti-growth hormone reacted with the nuclear membrane and adjacent cytoplasm of many cells of different distribution from all of the above; these cells were not identified. This reaction could not be inhibited but it was abolished by absorption of the conjugate with ACTH, pituitary TSH or growth hormone.

None of the conjugates gave staining in human kidney, liver or adrenal. All of them gave bright granular staining of the cytoplasm of acidophils in the ox pituitary (Figure 48). The same cells were stained by a labelled antiserum to ox growth hormone (For details of the preparation of this antiserum see Appendix, pp. 196). The reaction in the ox pituitary with all of these conjugates could neither be inhibited nor absorbed. The unlabelled anti-ox growth hormone did not give a cross-reaction with any of the human hormones in precipitin or gel-diffusion tests, nor did the labelled sample give staining in human pituitaries.

DISCUSSION

These results raise two questions: (1) the specificity of the antisera obtained by immunisation with the preparations of hormones and (2) the reliability of the fluorescent antibody method for localising normal tissue components.

Hormone-specific Antibodies

The initial object of this work was the demonstration by the fluorescent antibody method of the site of formation of ACTH, TSH, growth hormone and gonadotrophin in the human pituitary. This was dependent upon the production of hormone-specific antisera. There is very little evidence that this has been achieved. Thus the precipitin test between ACTH and anti-ACTH gave a higher titre than similar tests with other antisera and their homologous antigens (Table ~~xiii~~) and the gel-diffusion/

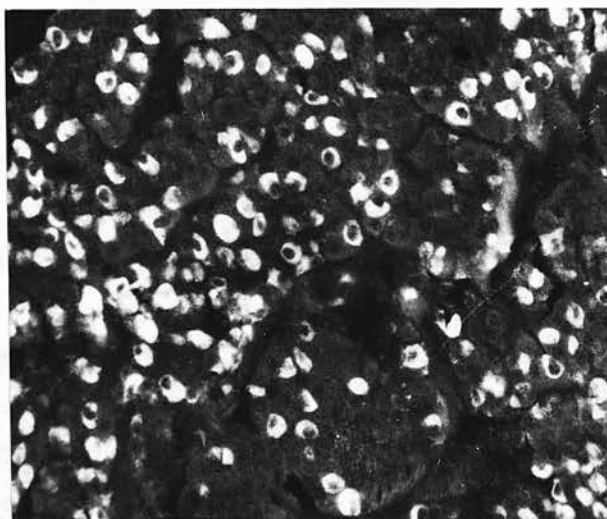


Figure 48. Fresh-frozen section of ox pituitary treated with fluorescein-labelled anti-human ACTH. Fluorescent material is in the cytoplasm of acidophils. Inhibition and absorption tests had no effect on this staining. (x 300)

diffusion test suggested a specific line between the same reagents. However, precipitin and gel-diffusion tests after absorption of the antiserum with the other pituitary extracts failed to confirm this result. The bio-assay performed after the precipitin test indicated that the hormonal activity of the ACTH preparation was reduced by exposure to the antiserum. This could be due to the presence of a hormone-specific antibody, but it might also occur if the hormone were attached to an antigenic carrier and were precipitated with that protein. The localisation experiments failed to show the presence of any specific antibody. No antibody specific to either preparation of TSH was demonstrated in the absorption-precipitin or gel-diffusion tests. The absence of any cross-reaction between these two extracts of the same hormone and their respective antisera is further evidence that neither antiserum contained a hormone-specific antibody. No evidence of a hormone-specific antibody to post-menopausal gonadotrophin or to growth hormone was obtained.

Although no clear-cut evidence of hormone-specific antibodies was obtained, all of the preparations used were antigenic. The results of the serological tests indicate that there was an antigen common to the three pituitary extracts and another antigen, or antigens, common to all of the urinary extracts. It would be interesting to know whether these antigens are related to the biologically active components of the extracts, say as carrier proteins. If this is so then further purification of the extracts to remove these proteins might result in preparations which have increased hormone content but which may be no longer antigenic.

Previous studies on the ability of the anterior pituitary hormones to stimulate the production of hormone-specific antibodies have also led to conflicting results. Thus Chase (1949) claimed that a preparation of ACTH which was homogeneous by electrophoresis and ultracentrifugation and in which there was no evidence of growth, gonadotrophic, thyrotrophic or lactogenic hormonal activity with the bio-assay methods used, was capable of provoking precipitating antibodies in mice. These antibodies neutralised the biological activity of the hormone preparation. Gordon (1949) was unable to produce precipitating antibodies in rats immunised with hog ACTH, though some/

some of the animals became refractory to the hormone. The antiserum used by Marshall (1951) was produced by immunising adrenalectomised rabbits with hog ACTH and it precipitated 10-20 per cent of the biological activity of a preparation of the hormone 120 times the standard potency. Morrison et al. (1952) found that a preparation of hog ACTH which was electrophoretically homogeneous (Fishman, 1947) was not antigenic in intact rabbits. Allergic reactions have occurred in human patients after treatment with ACTH but, in most instances, it has not been established whether this was due to the hormone itself or to an impurity. However, in the patient studied by Feinberg et al. (1951), there was evidence that the reaction was due to the hormone. On the other hand, Clayton et al. (1952) demonstrated that a complement-fixing antibody had no effect on the biological activity of the ACTH preparation used and that there was no correlation between the antibody level and the clinical response. Recent clinical studies have suggested that 'purified' hog ACTH may not be antigenic. Thus, Perkoff et al. (1955) performed Prausnitz-Kustner passive transfer tests with the serum of a patient who had developed a severe anaphylactic reaction to hog ACTH. Positive reactions were obtained with the hog ACTH used in treatment, with another preparation of hog ACTH and one of sheep ACTH, but no reaction occurred with 'highly purified' oxycellulose hog ACTH. West (1956) found that patients with acquired resistance to the effect of various ACTH preparations, including some 'purified' and 'improved' corticotrophins, responded clinically and by increased steroid excretion to corticotrophin A₁ (Dixon and Stack-Dunne, 1955). Some of the patients had, in addition, developed local 'allergic' reactions to the earlier preparations but showed no such reactions with corticotrophin A₁.

Similar studies of growth hormone (Elberg and Li, 1950; Ferguson and Boyden, 1951; Morrison et al., 1952), pituitary gonadotrophin (Chow, 1942; Van Dyke et al., 1950; Steelman and Bunting, 1955) and thyrotrophic hormone (Eichbaum and Kindermann, 1936; Rowlands and Young, 1938) have also produced conflicting results.

There are several reasons why these earlier results are difficult to interpret. The animal source, the method of extraction/

extraction, the purity of the hormone preparations and the bio-assay methods used have varied. In some instances there has been no indication of the biological activity or purity of the preparation. The animal species used for the production of the antisera and the methods for determining the antibody content of these sera have varied. Many previous workers have used one, or at most two, hormone preparations from the same species. The hormone-specificity of the antisera has been judged from cross-reactions between the antisera and serum protein or extracts from other organs of the species from which the hormones themselves were extracted and, sometimes, from cross-reactions between the antisera and preparations of the same hormone from different species. Tests for cross-reactions between the antisera and other hormone extracts derived from the same species as the hormone in question have rarely been made. The results presented here indicate that, in studies of anterior pituitary hormones, it is essential to test the antisera against different hormones from the same source of the same species before concluding that hormone-specific antibodies are present.

Although some believe that the serum component responsible for precipitation of a hormone is also responsible for neutralising its biological activity (Twombly, 1936; Wolfe et al., 1945; Chase, 1945), others believe that these two properties of an antiserum are unrelated (Bachman, 1935; Rowlands, 1937-8; Zondek and Sulman, 1942) and use the terms 'precipitating antibody' and 'neutralising antihormone' to describe them. Clear-cut evidence that these factors can be unrelated has been produced by Van den Ende (1941), who worked with chorionic gonadotrophin. Such information is not available for any of the anterior pituitary hormones, so that results based only on the ability of an antiserum to neutralise the biological activity of the hormone may not necessarily indicate the presence of hormone-specific precipitating antibodies.

Tissue Localisation

It would appear to be of little value to attempt tissue localisation studies with antisera such as those produced in the present study. However, the results obtained with the labelled samples of these sera are such as to make the interpretation of any/

any future work difficult. Despite the lack of proof of a hormone-specific antibody, staining was obtained with anti-ACTH in the very site in which it was to be expected if the serum had contained such an antibody. The failure to reproduce the staining with anti-ACTH after some months is puzzling but could be explained if this serum had contained a hormone-specific antibody, for Sulman (1939) and Chase (1945) have shown that hormone-specific antibodies to gonadotrophins deteriorate on storage. Staining with the other antisera also occurred in the cytoplasm of cells in the anterior pituitary. Tests to establish the nature of this staining merely confirmed the results of the serological tests. Coons (1956) has suggested that quantitative absorption of labelled antisera to a pituitary hormone with extracts containing differing amounts of the hormone might remove unwanted reactions and establish the specificity of staining. It is doubtful if any of the hormone preparations currently available are sufficiently pure or contain a sufficiently high concentration of the biologically active hormone to be effective in such a procedure.

The studies reported here were commenced in the belief, founded on the previous publications quoted, that the fluorescent antibody technique could be used to demonstrate the precise cellular localisation of anterior pituitary hormones in man. The results indicate some of the pitfalls encountered and serve to indicate the necessity for careful immunological control of such studies. Clearly, much further work is required before such normal tissue components as the anterior pituitary hormones can be localised in their cells of origin.

SUMMARY

1. Antisera have been produced to adrenocorticotrophic hormone, thyrotrophic hormone and growth hormone extracted from human pituitaries and to gonadotrophin and thyrotrophic hormone extracted from human urine.
2. Precipitin, absorption-precipitin and gel-diffusion tests have been performed with these antisera.
3. No conclusive evidence was obtained of the presence of a hormone-specific antibody in any of the antisera.
4. Cross-reactions occurred indicating the presence of an antigen common to those extracts prepared from the pituitary and another/

another antigen, or antigens, common to those extracts prepared from urine.

5. Despite the absence of hormone-specific antibodies, fluorescein-labelled samples of the antisera gave staining in the cytoplasm of cells in the anterior pituitary but did not stain other tissues.

SECTION V

GENERAL DISCUSSION

This work has exposed and emphasised some of the difficulties involved in the application of the fluorescent antibody technique to the localisation of tissue components. They are best discussed in relation to the various steps in the technique, under the following headings:

- a) preparation and purity of antigens,
- b) analysis of immune sera,
- c) effects of conjugation and
- d) specificity of staining.

Certain additional problems associated with studies on auto-antibodies will then be discussed. This section of the thesis ends with suggestions for further work arising from the experiments already conducted.

PREPARATION AND PURITY OF ANTIGENS

It is obviously ideally desirable to immunise animals with a purified antigen of known chemical composition, preferably a single substance. This is not often possible with tissue components. It is easier if the antigen is insoluble and more resistant to mild treatment than other components of the same tissue than it is with a soluble antigen. Thus, previous work on nephrotoxic nephritis, reviewed in Section II, pp. 5 - 7 led to the present work on basement membrane and reticulin, using glomeruli rather than whole kidney as the antigen. Isolation of glomerular basement membrane can be achieved by physical — ultrasonic vibration (Krakower and Greenspon, 1951) — or chemical methods — dilute sodium hydroxide (Little and Kramer, 1952) or tryptic digestion (Milazzo, 1957). Considerable purification of complex solutions, such as plasma proteins can be achieved by precipitation under suitable conditions (Cohn et al., 1950), chromatography on ion-exchange resin columns (Sober et al., 1956) or various forms of electrophoresis.

It must be noted, however, that methods for the extraction and purification of tissue components may be adequate for purely chemical or biological work but unsatisfactory for immunological studies. This is particularly so if the extraction is not accompanied by histological examination to check the source of the material, as in the case of tryptic digests of kidney and glomeruli and the pituitary hormone preparations. Furthermore, the extraction procedure may itself alter or destroy the antigenicity of the material. The chondroitin sulphate/

sulphate preparation used here seems to have been altered in such a way that the antigen present stimulated sera with extensive cross-reactions but no specific antibody.

Denaturation is a constant problem in all procedures for the isolation of plasma proteins. Thus, the plasma and urinary mucoproteins isolated by Anderson and MacLagan (1955) have no immunological activity (Grant, 1958).

ANALYSIS OF IMMUNE SERA

The work with the anterior pituitary hormones emphasises the need for careful and detailed preliminary serological tests before labelling antisera. Marshall (1951), whose work with hog ACTH stimulated the present work, did not report tests for cross-reactions with other pituitary hormones. In the light of the experiments now reported his conclusions must be accepted with considerable reserve. In a later study, Marshall (1954b) claimed to have localised three pancreatic enzymes within the acinar cells. His antigens were crystalline preparations but no preliminary serological tests were reported. The distribution of the staining with the labelled antisera to two of these enzymes was identical; staining with the third antiserum occurred in these and other sites. Without proper preliminary tests these observations are meaningless.

Antisera should be tested against all known antigens in the preparations used for immunisation and against all possible cross-reacting antigens or haptens. The value of the Ouchterlony gel-diffusion method in such tests can be judged from the work of Vaughan and Kabat (1954) who detected a previously unidentified antibody in antisera to egg albumin by its use: the antigen responsible constituted less than 1 per cent of the total protein in egg white. Even with an insoluble antigen, such as basement membrane, preliminary serological analysis is desirable. Two unwanted antibodies, namely to red cells and to serum proteins were detected in the antisera to kidney used here. More detailed analysis of such antisera is probably necessary in view of the close association of endothelial cells with glomerular basement membrane and with reticulin in many tissues and of the probable importance of cellular antigens in nephrotoxic nephritis. Complement-fixation tests or the agglutination tests described by Milazzo should give the necessary/

necessary information about antibodies to the insoluble antigens. This will be essential in studying the antigenic relationship of different types of reticulin (see later). In addition to the procedures used in the present work with soluble antigens, the immuno-electrophoretic method of Grabar and Williams (1955) is of great value, particularly in the analysis of antisera to plasma (Gell, 1957) or urinary (Grant, 1957) proteins. The analytical properties of both gel-diffusion and immuno-electrophoresis can be increased by the use of stains for the identification of immune precipitates containing lipoproteins and glycoproteins (Uriel and Grabar, 1956).*

When the various antibodies present in an immune serum have been identified, it is frequently possible to absorb out those which are not required. Thus, in the present work the antibodies to red cells and serum proteins were removed from the antikidney sera. Sera should not be used for labelling if the antibodies present cannot be identified, as in the experiments with the tryptic digests, or the unwanted or cross-reacting antibodies cannot be removed, as in the experiments with chondroitin sulphate and the pituitary hormones. The extent to which such absorptions may have to be carried is shown in the work of Humphrey (1955), who demonstrated the immunological identity of platelets and megakaryocytes. The specificity of the reaction was apparent only after the antiserum to guinea-pig platelets had been absorbed repeatedly with red cells, liver, kidney, spleen and polymorphs. Henry and Van Dyke were able to reduce the number of detectable antibodies in their anti-ICSH sera from six to one by appropriate absorptions. They then demonstrated that the absorbed serum inhibited the biological activity of the hormone in question. This is an additional test which is available in immunological studies on hormones and enzymes and it is essential if the immunising preparation contains impurities. The work of Gitlin et al. (1953) on the distribution of homologous plasma proteins in human tissues also indicated that useful information can be obtained with antisera containing unwanted antibodies, provided that careful preliminary tests are performed. This was the first of many articles, from this and other groups of workers on the localisation of plasma proteins with the

*Henry and Van Dyke (1958) have obtained useful information about the antibodies produced by the injection of biologically pure sheep interstitial cell stimulating hormone (ICSH) from a combination of continuous paper electrophoresis and gel-diffusion tests. No less than six antibodies were detected

ATTACHMENT AND PROPERTIES OF THE FLUORESCENT LABEL

Coons (1954), in a review of different types of label for immuno-histochemical work, defined the ideal properties of such a label. It should have no effect of its own on the antibody, the reaction between antigen and antibody, or on the tissues; it should remain firmly attached to the antibody molecule during and after the reaction; it should be readily identifiable in the tissue after the reaction has occurred and be distinguishable from heterologous material. Fluorescein isocyanate fulfils most of these criteria but it has two drawbacks -- the labelling procedure causes a fall in the titre of the antibody, and some fluorescein is adsorbed on to the protein rather than chemically bound to it.

Both of these drawbacks were encountered by Coons himself, who found that a specific reaction was demonstrable with some antisera before conjugation yet no staining occurred with the labelled sample, although the specific antigen was known to be present in the tissue (Coons et al., 1942). Fabius (1955) has shown that the titre of rabbit anti-human globulin serum may fall from 1/2048 to 1/8 (serum dilution) on conjugation and that the procedures accompanying the conjugation may cause a four- to eight-fold fall in titre. Van Doorenmaalen (1957) found that the titre of anti-frog lens serum fell from 1/20,000 to 1/1280 (antigen dilution) on conjugation. This observation is particularly significant, for it suggests an alteration in the avidity of the antibody, which is much more serious than a fall in the serum titre. The latter can be overcome by concentrating the conjugate and both effects by using the indirect method (Weller and Coons, 1954). This is, in effect, a Coomb's test with a labelled anti-globulin serum and it increases the sensitivity of the method by about ten-fold (Coons, 1956). The tissue is treated with unlabelled immune serum, or globulin, washed and then treated with a labelled antiserum to the globulin of the species in which the immune serum was prepared. This second serum, which can be obtained in high titre and is thus less affected during labelling, reacts with the precipitated immune globulin and can be visualised in the usual way. The efficacy of this method was shown in some experiments, not recorded here, with rabbit anti-mouse kidney sera. No reaction occurred when the labelled immune serum was used, but, when unlabelled anti-mouse kidney serum was followed by labelled goat/

goat anti-rabbit-globulin serum, bright staining occurred.

Adsorbed fluorescein is removed by absorbing the labelled immune serum with an unrelated tissue powder before use on the tissue sections (Coons and Kaplan, 1950). Prolonged storage of the labelled antiserum is associated with dissociation of some more fluorescein, so that further absorption becomes necessary. There is considerable loss of fluid at each absorption with the acetone-dried tissue powders used by Coons, so that, when labelled antisera are prepared in small quantity, this may become a serious nuisance. Kaplan (1958a) has recently described wet tissue preparations which are suitable for absorption and should to some extent overcome this difficulty. The fluorochrome lissamine rhodamine RB200, introduced by Chadwick et al. (1958), appears to be less liable to this disadvantage than fluorescein and has given satisfactory results with the anti-human-glomerulus serum.

SPECIFICITY OF STAINING

Inhibition and absorption tests, such as those described on pp. 8-11, should be sufficient to establish the specificity of staining in most instances, provided adequate preliminary tests have been performed with the unlabelled antisera. The results in the present experiments with pituitary hormones emphasise the necessity for establishing the specificity of staining and show, also, that non-specific staining may occur in sites where specific staining is to be expected. There~~is~~, at present, no explanation for this troublesome artefact, which has not been described by other workers but will have to be kept in mind in future work. Occasionally, as in the work of Scott (1957), discussed in Section II, pp. 45-47, it may be difficult to design suitable absorption tests but cross-inhibition tests with antisera to closely related antigens can be used.

Still further precautions are necessary in some studies, particularly those of plasma proteins. Thus Dixon et al., (1958) believe that much of the gamma globulin demonstrable in the lesions of experimental serum sickness accumulates non-specifically as part of an inflammatory exudate. They suggest that this may also be true of gamma globulin in human lesions, such as amyloidosis and glomerulonephritis, and have shown that the use of more than one labelled antiserum is necessary to obtain unequivocal results (Vazquez and Dixon, 1957). Considerable care is/

is required in the use of the indirect method when the immune serum has been injected in vivo (Mellors, Siegel and Pressman, 1955) rather than applied to tissue sections, as described above. Normal (non-immune) serum proteins take up a characteristic distribution in the tissues after intravenous administration in an animal of a different species (Coons et al., 1951).

Presumably similar fractions of an immune serum similarly injected will become distributed in the same way, in addition to the specific distribution of the gamma globulin at sites where it reacts with antigen. If the in vivo method is used it is desirable to inject purified immune globulin, to use purified gamma globulin for the production of the anti-globulin serum and to establish that neither the immune globulin nor the anti-globulin serum contains unwanted antibodies. In the experiments of Mellors, Siegel and Pressman and of Ortega and Mellors (1956) the immune globulins injected were impure and it is not clearly established that the anti-globulin sera used in these and other studies (Mellors, Arias-Stella, Siegel and Pressman, 1955) were specific. This difficulty is less serious when the indirect method is used entirely for in vitro work, as in the studies reported here in human nephritis and anaphylactoid purpura, but it is still preferable to use a pure anti-globulin serum, as was done here (see Figs. 44 and 45, p.108).

STUDIES ON AUTO-ANTIBODIES

The present work has emphasised the need for careful analysis of all reactions between human sera and tissue extracts before they can be interpreted as immunologically significant. The criticisms of previous work on nephritis (Section III, pp.114-9) apply also to the extensive literature on other diseases, most of which has been reviewed in a recent book (Miescher and Vorlaender, 1957). It is essential that strict criteria, such as those suggested by Witebsky et al. (1957), should be fulfilled before the presence of auto-antibodies is claimed or auto-immunisation implicated in the pathogenesis of a disease. By these criteria, the only human diseases in which auto-antibodies have been demonstrated are paroxysmal haemoglobinuria and some other forms of acquired haemolytic anaemia, systemic lupus erythematosus and Hashimoto's disease. The last-named is the only one in which similar lesions have been produced in animals by/

by auto-immunisation.

It is more convenient and economical to use the indirect fluorescent antibody method in studies on auto-antibodies, for only one serum -- anti-human-globulin -- need be labelled, whereas each individual human serum must be labelled if the direct method is used. The use of the indirect method becomes essential in circumstances where antibodies may be present in low titre in the human sera (see above). This may explain the inability of Hill and Cruickshank (1950) to obtain staining of nuclei, using labelled sera from cases of systemic lupus erythematosus, whereas subsequent workers have been successful with the indirect method (Holborow et al., 1957; Holman and Kunkel, 1957; Friou et al., 1958). Indeed, the direct method may be unsuccessful even when antibodies can be demonstrated in high titre by other methods, as in Hashimoto's disease. Thus, both White (1958) and the author have been unable to get staining with labelled samples of patients' sera which gave bright staining in the indirect method with a labelled rabbit anti-human-globulin serum. Non-specific staining may be a considerable nuisance in such studies, particularly when an anti-globulin serum is used on tissues of the species from which the globulin was derived. However, Kaplan (1958a) has recently introduced a manoeuvre which abolishes such non-specific staining when labelled anti-rabbit-globulin is applied to rabbit tissues and which should be applicable to labelled anti-human-globulin.

SUGGESTIONS FOR FURTHER WORK

There is considerable scope for further work with the fluorescent antibody technique on some of the problems studied already and on many other aspects of tissue antigens.

The suggestion from Scott's work (1957) that basement membrane and reticulin may be antigenically distinct, is supported by the results of Lillie (1952a), with the allochrome procedure, and of Goodman et al. (1955), in studies on the antigenicity of various components of the canine kidney. As indicated already, however, Scott's results are not clear-cut and have not been confirmed. In further studies on the antigenic relationship of basement membrane and reticulin, attention should be paid also to the argyrophilic, trypsin-sensitive and collagenase-resistant fibres described in the ovary by/

by Robb-Smith (1952) and in the ciliary region of the vitreous body by Bembridge et al. (1953). Some of this work has been started already and labelled antisera to human arterial reticulin and to human synovium are available; these and anti-human-glomerulus serum have been labelled with both fluorescein isocyanate and lissamine rhodamine RB200 (Chadwick et al.). It is also proposed to use the anti-human-glomerulus serum to study the basement^{membrane} of the thyroid in some detail, particularly in relation to Hashimoto's disease for, although it is accepted that auto-antibodies to thyroglobulin are responsible for the progressive thyroid damage, the pathogenesis of the initial stage is unknown. There seem to be two possibilities -- inability to confine thyroglobulin within the acini or lack of a means of destroying such thyroglobulin as does escape during the physiological activity of the gland. The former could be the result of maldevelopment of, or damage to, the basement membrane of the acini. The immuno-histochemical method appears to be the most satisfactory way of tackling this problem, particularly as it is superior to other methods for demonstrating the thyroid basement membrane (see Section II, p. 51-52).

The results of the experiments with collagen are sufficiently encouraging to suggest that further attempts should be made to obtain an antibody, which can be used either by the direct or by the indirect method. The choice of an antigen for these experiments is more complicated now than it was when the earlier experiments were conducted. Several forms of soluble collagen have been described and there are differences of opinion about their relationship with mature collagen (see Tunbridge et al., 1957). Bowes et al. (1957) have shown that the protein composition of these soluble collagens differs slightly and Fessler (1957) has obtained three different fractions from one of the soluble collagens. It will thus be necessary to use several, if not all, of these soluble collagens in future work. In the meantime, rabbits are being immunised with preparations from human granulation tissue, on the assumption that the fine argyrophilic fibres found there are immature collagen, rather than true reticulin. However, there is evidence that regenerating connective tissue contains both types of fibre (Robb-Smith, 1957; Windrum, 1958) and the capillaries in such tissue certainly contain true reticulin, so that very careful analysis of the sera will/

will be necessary. Preliminary results of other experiments, in which rabbits have been immunised with mouse kidney or mouse granulation tissue suggest that both antisera contain antibodies to reticulin: no antibody to collagen has been demonstrated so far. It is hoped to study pathological changes in human reticulin and collagen if good antisera to the various fibres can be prepared, but there is still much preliminary work to be done before such studies can be contemplated.

The need for a means of distinguishing different sulphated mucopolysaccharides has already been discussed (Section II, pp. 77-79). It is proposed to use some of the newer preparations for immunisation and arrangements have been made to obtain some pig chondroitin sulphate from Dr. Helen Muir. The attempts to prepare an antibody to heparin should also be repeated. A specific means for identifying hyaluronic acid in the tissues is not at present available and would be of great value in studies of connective tissue mucopolysaccharides. The rather meagre information available suggests that hyaluronic acid may not be antigenic. Thus Humphrey (1943), using potassium hyaluronate from human umbilical cords, obtained no antibody to the mucopolysaccharide moiety of hyaluronate-serum complexes. Cruickshank (1950) immunised rabbits with human rheumatoid synovial fluid. After the antisera had been absorbed with normal human serum they failed to give any reaction with the synovial fluid. These results may not be significant as the hyaluronic acid of rheumatoid synovial fluid is known to be degraded (Ropes et al., 1947). This may also be true of extracted hyaluronic acid. Further attempts, using undegraded synovial or umbilical cord mucin with adjuvants are indicated.

It is not proposed to pursue the pituitary hormone problem further until much purer preparations are available. Biological purity, as in the preparation of ICSH, used by Henry and Van Dyke (see above) is not sufficient for immunological work, for antigenicity may be due to impurities. An absorbed serum, such as they obtained, with a single detectable antibody capable of inhibiting the hormonal activity of a biologically pure preparation, could be used for tissue localisation studies provided it gave no cross-reaction with other purified hormones extracted from the pituitary of the same species. There seems little likelihood of obtaining sufficiently pure preparations from/

from the human pituitary for a long time.

There is obviously a need for further work on basement membrane and cellular antigens in nephrotoxic nephritis. However, this wholly experimental procedure can have no counterpart in human disease and it is not proposed to undertake any such experiments. It has been suggested (Section III, p. 122) that, if auto-immunisation plays any part in human nephritis, the tonsil is the most likely source of both antigen and antibody. However, the present results and the absence of a reproducible experimental model, despite repeated attempts, do not make this an attractive hypothesis. On the other hand, recent work on anaphylaxis suggests more likely possibilities. Thus Rich (1956) has shown that all the lesions of human glomerulonephritis can be produced in rabbits by hypersensitivity to foreign proteins and the importance of soluble antigen-antibody complexes in anaphylactic shock and serum sickness have been demonstrated by Germuth and McKinnon (1957) and Dixon et al. It may be that streptococcal antigens and their antibodies occur in similar complexes in human glomerulonephritis. Alternatively, Kaplan (1958b) has shown that streptococcal M protein localises in the glomerulus after injection into the mouse: the streptococcal types used included those shown by epidemiological studies to be nephritogenic (Rammelkamp, 1955-56). If this occurs in man, then the reaction between this protein and its antibody might result in nephritis. Attempts to demonstrate streptococcal antigens in human kidneys have so far been unsuccessful (Dixon, 1958) but should not be abandoned.

In conclusion, it can be said that much useful information should be obtained from the further application of the fluorescent antibody technique to the localisation^{of tissue antigens}, provided that the difficulties and weaknesses of the method are appreciated and that careful control is maintained at all stages.

SUMMARY

Problems involved in the use of the fluorescent antibody technique for the localisation of tissue components are discussed.

The need for pure tissue extracts for the immunisation of animals is emphasised and some of the dangers of extraction procedures are described.

The/

The dangers of inadequate preliminary analysis of immune sera are indicated. The need for accurate identification of all antibodies in an immune serum and methods for achieving this are discussed. Appropriate absorptions can be used to get rid of unwanted antibodies and examples are given of results obtained with such purified antisera.

Measures to counteract the fall in antibody titre caused by conjugation with fluorescein and the presence of adsorbed, rather than chemically conjugated, fluorescein are discussed, including the indirect fluorescent antibody method.

Precautions necessary to establish the specificity of staining with the direct and indirect methods are described.

Problems peculiar to studies on auto-antibodies are discussed.

Suggestions are made for further work arising from the present experiments.

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Rabbits were immunised with rat kidney, glomeruli or lung, the resulting immune sera absorbed with rat erythrocytes and serum and then conjugated with fluorescein isocyanate. The labelled antisera reacted with basement membrane and reticulin throughout the animal and with sarcolemma and neurilemma. No reaction occurred with collagen fibres, elastic fibres, cartilage matrix or ground substance. Species cross-reactions occurred only in the mouse. Similar results in human tissues were obtained with fluorescein-labelled antisera to human glomeruli. Species cross-reactions occurred in the rhesus monkey. The results are discussed in relation to those of other workers with different techniques on the relationship between basement membrane, reticulin and collagen. There is histological, histochemical, biochemical, biophysical and now immuno-histochemical evidence that basement membrane and reticulin are closely related, if not identical, whereas collagen is a separate entity.

Preliminary experiments have confirmed the antigenicity of collagen from rat and human tendon. Localisation experiments with fluorescein-labelled antisera were unsuccessful.

Attempts to produce a specific antibody to human chondroitin sulphate were unsuccessful. The extracted material was antigenic, but the antisera gave extensive cross-reactions, particularly with blood-group substances.

Tryptic digests of human kidney and glomeruli were antigenic in rabbits. The antisera reacted with saline extracts of kidney as well as with the tryptic digests. Localisation experiments with fluorescein-labelled antisera were unsuccessful. The results, together with those of other workers on nephrotoxic nephritis, suggest that much of the material in tryptic digests is derived from cells, rather than basement membrane, and that the relative importance of these two kidney components in the production of nephrotoxic antisera requires further investigation.

Sera and eluates from kidneys obtained at autopsy, from cases of acute glomerulonephritis and controls have been examined for antibodies against several extracts of normal human kidney by precipitin, agglutination and complement fixation tests. The direct and indirect fluorescent antibody techniques were used in attempts to demonstrate tissue-localising antibodies in the sera and eluates. Reactions occurred in complement fixation tests with nephritic and control sera and eluates against tryptic digests of/

of kidney or glomeruli and were shown to be non-specific. No evidence of specific antibodies to kidney components was demonstrated. The results are discussed in relation to those of other workers on experimental nephritis and on auto-antibodies in human diseases. It is concluded that there is no evidence that auto-immunisation to kidney is responsible for acute glomerulonephritis in man.

A similar investigation in anaphylactoid purpura showed no evidence of a specific antibody to kidney or blood vessel components.

Immunisation of guinea-pigs and rabbits with poliomyelitis vaccine revealed no evidence of monkey kidney antigens. A case of nephritis, which commenced shortly after vaccination, is reported; no evidence of auto-immunisation to kidney was found.

Rabbits were immunised with several human anterior pituitary hormones. The resulting immune sera gave extensive cross-reactions in precipitin and gel-diffusion tests. Similar tests after absorption of the sera revealed no evidence of hormone-specific antibodies. Nevertheless, fluorescein-labelled samples of the antisera reacted with the cytoplasm of cells in the anterior pituitary. Previous work on hormone-specific antibodies is critically discussed.

Problems involved in the use of the fluorescent antibody technique for the localisation of tissue components are discussed in relation to the present work and that of other authors. Suggestions are made for further work arising from the present experiments.

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ACKNOWLEDGEMENTS

The early stages of this work were made possible by the award of a Griffith Research Fellowship by the University and the Griffith Research Scholarship in Immunology. Later on, a grant was received from the Griffith Hospital Endowment Research Fund.

I wish to thank Professor Sir Stanley Davidson, D.Sc., F.R.S., for his criticism and for his helpful suggestions and for his criticism of the work. I wish, particularly to thank Dr. Alan Macmillan, who passed on the knowledge of the fluorescent antibody technique which he had acquired previously from Dr. A.L. Coombs and Dr. Frank Roy, who labelled so many of the antisera.

ACKNOWLEDGEMENTS

Thanks are due to the following clinicians for samples of sera and for access to their laboratories: Dr. F.A. Alexander, Dr. M. Leslie, Dr. A.D. Brown, Dr. J. Macdonald, Dr. J.A. Douglas, Prof. Sir Stanley Davidson, Prof. A.D.B. Ellis, Dr. J.D. Porter, Dr. E.R. French, Dr. A.L. Macmillan, Dr. J.B. Hutchinson, Dr. A.E. Innes, Dr. L.C. Lewis, Dr. C.J. Langford, Prof. V.A. Mackay, Dr. P. Macdonald, Dr. R. Macdonald, Dr. W.D.B. Scott, Dr. T.R.B. Todd, Dr. A.W. Wright and Dr. J.B. Wright.

Dr. G.D. Colman carried out the ultraviolet absorption of collagen; Dr. G.L. Gardner prepared the acid-soluble salt collagen; Dr. C. Wilson prepared and examined a sample of this for electron microscopy; Dr. A.B. Roy prepared the known chondroitin sulphate and most of the tryptic digests; Prof. V.L.J. Rogers prepared additional tests with the antisera to chondroitin sulphate; Miss Mary Gardner and Dr. C. Roy did protein estimations and electrophoresis of the eluates; Dr. G.L. Scott and Dr. J.B. Debus performed bio-assays on hormone preparations. To all these thanks are due.

Materials were supplied as follows: bovine chondroitin sulphate by the State Biological Inst., Panters, Cheshire; heparin by Harte Pure Drug Co., Nottingham; chondroitin lactate by the Ministry of Agriculture, Farnham; Crill 15 by Messrs Crill Ltd., Smith, Leeds, Texas; poliovaccine sulphate fluid by Dr. V. Wood, Hialeah, Fla.; BMD-1, Froggill, New York and BMD-2 by/

ACKNOWLEDGEMENTS

The early stages of this work were made possible by the award of a Nuffield Research Fellowship in Rheumatism and the Hastilow Research Scholarship in Rheumatism. Later on, a grant was received from the Scottish Hospital Endowments Research Trust.

I wish to thank Professor Sir Stanley Davidson, Dr. J.J.R. Duthie and Professors A.M. Drennan, G.L. Montgomery and T. Symington for providing facilities and for encouragement and criticism at different stages of the work. I wish, particularly, to thank Dr. Alan G.S. Hill, who passed on the knowledge of the fluorescent antibody technique which he had acquired personally from Dr. A.H. Coons, and Dr. Lynda Roy, who labelled so many of the antisera.

Thanks are due, also, to the following clinicians for samples of sera and for access to case notes: Dr. W.A. Alexander, Mr. W. Beattie, Mr. A.A. Bonar, Dr. J.D.S. Cameron, Dr. D.M. Douglas, Prof. Sir Stanley Davidson, Prof. R.W.B. Ellis, Dr. J.O. Forfar, Dr. E.B. French, Dr. A.R. Gilchrist, Dr. J.H. Hutchison, Dr. A.H. Imrie, Dr. I.C. Lewis, Mr. C.J. Longland, Prof. W.A. Mackey, Dr. P. Mackenzie, Dr. D. Nicholson, Dr. W.D.D. Small, Dr. T.R.R. Todd, Dr. A.W. Wright and Dr. J.H. Wright.

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Materials were supplied as follows: bovine chondroitin sulphate by the Evans Biological Inst., Runcorn, Cheshire; heparin by Boots Pure Drug Co., Nottingham; tubercle bacilli by the Ministry of Agriculture, Weybridge; Crill 16 by Messrs Croda Ltd., Snaith, Goole, Yorks; poliovaccine culture fluid by Dr. W. Wood, Glaxo Labs.; HMG20-A, Pregnyl, hog ACTH and DOCA by/

by Organon Laboratories Ltd; ox ACTH and growth hormone by Messrs Armour Ltd.; the extract of male urine by Dr. J.A. Loraine.

I am grateful to Drs. A.J.M. Fabius, I.R.W. Lominski and J.V. Wilson for permission to quote the results of their unpublished work.

The photomicrographs were processed in the Department of Medicine, University of Edinburgh, ^{the} Photomicrography Unit, Department of Pathology, University of Edinburgh and the Department of Pathology, Glasgow Royal Infirmary. I am grateful to Messrs. A.W. Wright, T.C. Dodds, P. Elliott and T. Parker for their help.

TECHNICAL PROCEDURES, ANIMAL PROTOCOLS

In order to avoid unnecessary repetition, the lay-out of this Appendix will follow the series of procedures involved in a complete localisation experiment.

PREPARATION OF ANTIGENS FOR INJECTION

Kidney

Rats (1 - 11) were anaesthetised with ether and the abdominal aorta exposed by a ventral incision. The vessel was tied off just above the bifurcation and a glass cannula inserted and tied in position between the bifurcation and the renal arteries. The inferior vena cava was incised and the animal perfused with 0.5 - 1 l. of saline. Blood was collected from the inferior vena cava during the first few moments of perfusion and the serum separated for later use. Usually the kidneys blanched quickly; those which did not were discarded.

The kidneys were emulsified by pressing through mono-metal gauze, washed with saline, spun and the supernatant fluid removed. The washing and spinning were repeated until the supernatant fluid was free of haemoglobin. The final supernatant fluid was discarded and the residue made up in a 10 - 20 per cent suspension in saline. This, and other particulate antigens, was ground in a Tenbroek tissue grinder (Griffith tube) before injection and 1/100,000 thiomersalate added as a preservative.

Monkey kidneys were obtained from the Physiology Department, University of Edinburgh. Human kidneys were obtained at autopsy as shown on p. 204. The monkey and human kidneys were homogenised with saline in a Waring Blender and then processed as above to give a 10 - 20 per cent suspension.

Glomeruli

Rat glomeruli were prepared from perfused kidneys (rats 17 - 20, 23 - 28). These were pressed with a glass rod through mono-metal gauze (145 mesh/inch). The resulting paste was collected and the glomeruli washed free of cellular debris with saline and spun at 2000 r.p.m. for three minutes; this process was repeated about six times. A 10 - 20 per cent suspension was used for immunisation.

Human glomeruli were prepared from kidneys obtained at autopsy as shown on p. 204. The original method was to remove the cortex from the medulla, homogenise in a Waring Blender, press through mono-metal gauze (80 mesh/ inch) and proceed as for the rat glomeruli. This method is tedious, particularly when large quantities of glomeruli are required, as for the preparation of tryptic digests, so various modifications were tried. The final method used was to freeze the kidney solid in the deep-freeze, separate cortex from medulla while partially frozen and mash in a vegetable mill. The resulting paste was suspended in a large volume of saline and allowed to stand for about one minute, during which the larger particles sedimented. The supernatant fluid was then decanted through mono-metal gauze (80 mesh/inch) and washed repeatedly with chilled saline: the glomeruli were allowed to settle either by standing at 4 C or by slow centrifugation (2000 r.p.m.). When the supernatant fluid was free of haemoglobin, the glomeruli were either packed by spinning, for storage in the deep-freeze, or processed for injection/

injection as above. Figs. 49 and 50 show a typical preparation.

Tryptic Digests of Kidney or Glomeruli

Digests 4, 6 and 7 were prepared from glomeruli and digest 8 from whole kidney by the method of Cole et al. (1951) and deproteinised by Sevag's method. Digests 9, 11 and 12 were prepared from whole kidney and digest 13 from glomeruli by the following modification. Kidneys were obtained at autopsy as shown on p.204 and glomeruli isolated as described above. 100 gm. of kidney or glomeruli were minced and extracted successively with 0.15 M NaCl, 1.5 M NaCl and 5 M KCl, using six 200 ml. portions of each solvent. The final residue was washed with 0.15 M NaCl, suspended in 0.15 M NaCl to give 100 ml., the pH taken to 8 with 0.1 M sodium borate and 1 gm. crystalline trypsin added. The mixture was incubated for 3 hr. at 37°C, adjusting the pH to 8 as required. The debris was spun off and washed with 25 ml. 0.15 M NaCl, washings being added to the original supernatant. The washing was repeated. The combined supernatant and washings was chilled and 3 vols. ethanol added, the mixture stood for 1 hr. and centrifuged at -10°C. The precipitate was dissolved in 10 ml. water. The solution was made 5 M KCl, stood overnight at 0°C, the precipitate spun off and discarded, and the supernatant dialysed overnight against running water.

Digests 4 - 11 were prepared by Dr. A.B. Roy, Department of Biochemistry, University of Edinburgh; digests 12 and 13 by the author.

Digest-Vaccine: Control Vaccine

Group-A, type-4 β -haemolytic streptococcus (N.C.T.C., Catalogue No. 8326) was incubated overnight in Todd-Hewitt broth. The culture was spun down and the organisms washed twice in saline. They were then suspended in 1 ml. of the digest and incubated for 1 hr. at 37°C. The tubes were spun and the organisms washed twice in saline, resuspended in saline to give an opacity of about 1×10^6 cocci/ml. and killed by heating at 56°C for 30 min. Fresh vaccine was prepared weekly.

The control vaccine was prepared in the same manner, using 1 ml. of saline in place of the tryptic digest.

Digest-Adjuvant Mixture

Digest 9 was mixed with adjuvants of the Freund type as follows:

kidney digest	5 ml.
Crill 16	2 ml.
liquid paraffin	5 ml.
tubercle bacilli	10 mgm.
	12 ml., containing 0.42 ml. digest/ml.

The digest and Crill 16 (an emulsificant supplied by Croda Ltd., Snaith, Goole, Yorks) were warmed to 45 - 50°C and emulsified in a Tenbroek tissue grinder. The liquid paraffin, containing the killed tubercle bacilli (supplied by Ministry of Agriculture, Weybridge), was warmed to the same temp. and added to the mixture, which was further emulsified.

Rat Lung

The lungs from rats 1 - 11 were homogenised in a Waring Blender/



Figure 49. Human glomeruli. Preparation used for immunising rabbits, isolation of tryptic digests and preparation of trypsinised glomeruli. Unfixed and unstained , wet preparation. (x80).

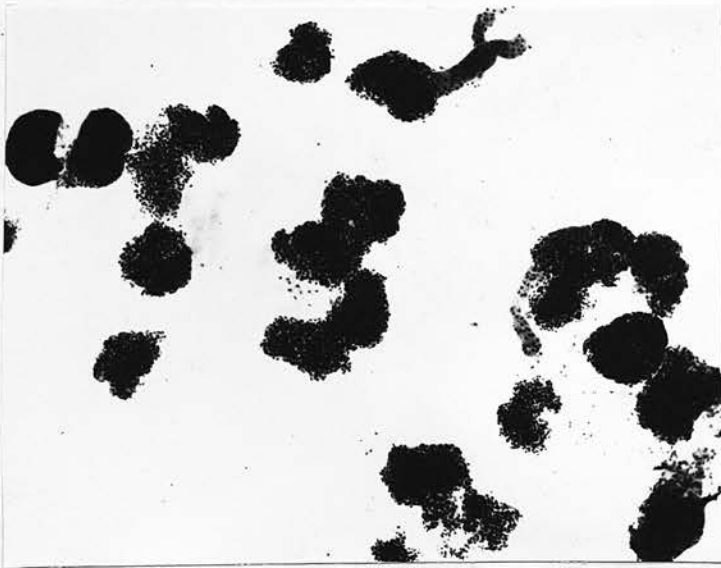


Figure 50. Human glomeruli. Preparation similar to Fig. 49. A few fragments of proximal convoluted tubule are present. Fixed and stained with methylene blue. (x60).

Blender, washed free of haemoglobin, as for the rat kidney, made up as a 10 - 20 per cent suspension and ground in a Tenbroek tissue grinder. Some difficulty was experienced in obtaining a homogeneous emulsion and large particles of tissue had to be discarded.

Rat and Human Serum Proteins

The proteins from whole serum were precipitated with 1 per cent alum/100 mgm. protein by the method of Heidelberger and Kendall (1935), the precipitate washed in saline and made up in a concentration of 5 or 10 mgm. protein/ml.

Human gamma globulin, from 10 ml. serum, was precipitated with sodium sulphate and mixed with Freund's adjuvants as follows:

gamma globulin	2.5 ml.
Crill 16	2.0 ml.
liquid paraffin	2.5 ml.
tubercle bacilli	5.0 mgm.
	6.5 ml.

Chondroitin Sulphate and Chondroitin Sulphate-Vaccine

The bovine material, isolated by the alkali method of Bray et al. (1944) and human material, isolated by the neutral potassium chloride method of Eindinder and Schubert from costal cartilages, were injected as 1 per cent solutions. Further samples of human chondroitin sulphate were prepared from two patients of known blood-group and supplied to Prof. W.T.J. Morgan. The sources of the costal cartilages are shown on p. 204.

Vaccines were prepared as described above, using the same strain of streptococcus and 1 per cent chondroitin sulphate in place of the tryptic digest.

Acid-soluble Collagen

Three preparations were made using a total of 24 rats. The tendons were extracted from the tail by cutting across each segment (separating distally) and pulling out with fine forceps. They were then finely cut with sharp scissors in a small amount of normal saline and transferred to a beaker containing approx. 100 ml of normal saline where they were stood at 4°C overnight. After washing in more saline, the pieces of tendon were transferred to 1/10,000 acetic acid, in which they swelled and became translucent. After 2 - 3 days the amount of acetic acid was gradually increased to 2 l. In this they were stood for a further indefinite period. Gentle mixing with a stirring motor at a temp. as near 0°C as possible was then performed and the material left to stand overnight. The gelatinous fluid was then transferred to 250 ml. centrifuge containers and stood at 4°C overnight. The following morning these containers were spun at 4°C for 1 hr. On removal from the centrifuge the material was found in two phases -- a compact, gelatinous mass (about 10 ml) at the bottom and the remainder a supernatant fluid. The fluid was aspirated off, the gelatinous material pooled and freeze-dried. A 0.1 per cent solution in 1/10,000 acetic acid was used for injection and, later, for complement fixation tests.

These preparations were made by Dr. D.L. Gardner, Pathology Department, University of Edinburgh.

Hormone/

Hormone Preparations

ACTH, TSH (both preparations), post-menopausal gonadotrophin and growth hormone (human and ox) were all used as alum-precipitates. Each extract was dissolved in saline and 5 per cent disodium hydrogen phosphate and 5 per cent potassium alum were added in the ratio of 4:3. The mixture was shaken, spun and the precipitate washed twice with saline and made up in concentrations varying from 0.6 to 2.0 mgm./ml.

Human growth hormone was also used with Freund's adjuvants, the mixture containing:

growth hormone	15.0 mgm.
saline	2.5 ml.
Crill 16	1.0 ml.
liquid paraffin	3.0 ml.
tubercle bacilli	6.0 mgm.
	6.5 ml., containing 2.3 mgm. extract/ml.

The method of preparation is given above.

PREPARATION OF ANIMALS

Adrenalectomy

This proved difficult, particularly on the right side, where the gland lies in the angle between the renal vein and the inferior vena cava, with the capsule of the medial aspect inseparable from the vena cava. Indeed, in some animals adrenal cortical tissue was found histologically in the wall of the vein. Nine rabbits were lost as a result of the operation before a satisfactory technique was evolved.

The final technique was as follows. Left adrenalectomy was performed, under nembutal supplemented by ether, through a left paramedian incision. The gland was mobilised by cutting through the parietal peritoneum and freeing it from the surrounding fat. The vessels were tied with double ligatures and cut. The gland was then excised in one piece. Little trouble was experienced on this side, though in one rabbit which survived to the end of the experiment (rabbit 122), the left kidney was eventually found atrophic, fibrosed and extensively calcified, though it appeared normal at the operation. Fourteen days later, the right adrenal was removed, under similar anaesthesia, through a subcostal incision. Allport's self-retaining retractor proved very useful in allowing access to the deep tissues. The adrenal was approached from the ventral aspect, if possible: if not, the kidney was mobilised and retracted forward. Veins in the retroperitoneal fat were often troublesome and had to be ligated. The adrenal was mobilised from the ventro-lateral or lateral aspect, the capsule incised, the gland removed piece-meal and the stump carefully cauterised. It was possible, in this way to remove at least three-quarters of the gland. Sulphonamide-penicillin powder was dusted into the deep part of the wounds and into the muscles. The animals were given procaine penicillin or streptomycin intramuscularly immediately after each operation and morning and afternoon for the next two days. 2 mgm. DOCA in oil (Organon) was given immediately after the second operation and on the next day; thereafter a salt block was left in the cage. Immunisation was begun 14 days after the second operation.

Thyroidectomy

Bilateral thyroidectomy was performed, under nembutal supplemented/

supplemented by ether. Occasional difficulty was experienced in differentiating the gland from adjacent muscles. Bleeding was often troublesome and was most easily controlled by cauterisation. One rabbit died the day after operation from asphyxia due to haemorrhage at the operation site. Another died 58 days after operation: no cause of death was found at autopsy. Sulphonamide-penicillin powder was dusted into the operation site and procaine penicillin given immediately and on the next day.

Castration

Male rabbits were used for immunisation with gonadotrophin, so that castration was easily performed through small scrotal incisions. No anaesthetic was used.

IMMUNISATION PROTOCOLS

Rabbits 42 - 44

Antigen: rat kidney suspension, intraperitoneally (ip.)

1st course: 1 ml. injected on 4 & 5/10/51, 11 & 12/10/51,
18 & 19/10/51, 25 & 26/10/51.

Dose of kidney 1 gm.

Bled on 1/11/51.

2nd course: 1 ml. injected on 2 & 3/11/51, 8 & 9/11/51,
15 & 16/11/51, 22 & 23/11/51.

Dose of kidney 1 gm.

Bled on 30/11/51 and exsanguinated on 3/12/51.

Rabbit 46

Antigen: rat lung suspension ip.

1st course: 1 ml. injected on 30 & 31/1/52, 6 & 7/2/52,
13 & 14 /2/52, 19 & 20/2/52, 4 & 5/3/52.

Bled on 10/3/52 and 18/3/52. Dose of lung 2 gm.

2nd course: 1 ml. injected on 2 & 5/4/52, 7 & 8/4/52, 10/4/52,
17 & 18/4/52, 24/4/52. Dose of lung 1.6 gm.

Bled on 1/5/52.

Rabbit 47

Antigen: rat glomerulus suspension ip.

1st course: 1 ml. injected on 18 & 19/2/52, 26 & 27/2/52,
4 & 5/3/52, 11 & 12/3/52. Dose of glomeruli 120 mgm.

Bled on 18/3/52.

2nd course: 1 ml. injected on 19 & 20/3/52, 25/3/52, 2/4/52,
8/4/52, 17/4/52.

Dose of glomeruli 75 mgm.

Exsanguinated on 1/5/52.

Rabbit 48

Antigen: human glomerulus suspension ip.

1st course: 2 ml. injected on 18 & 19/2/52, 26 & 27/2/52,
4 & 5/3/52, 11 & 12/3/52.

Dose of glomeruli 240 mgm.

2nd course: 1 ml. injected on 5/4/52, 7 & 8/4/52, 10/4/52, 17/4/52.

Dose of glomeruli 150 mgm.

Exsanguinated on 24/4/52.

Rabbit/

Rabbit 49

Antigen: rat serum protein (5 mgm./ml.) intravenously (iv.) on alternate days.

1st course: dose 1	0.5 ml. (19/2/52)
doses 2 & 3	1.0 ml.
doses 4-6	1.5 ml.
doses 7-10	2.0 ml.
doses 11-14	3.0 ml.
dose 15	4.5 ml. (21/3/52)
	<u>32.5 ml., containing 160 mgm. antigen.</u>

Bled on 1 & 2/4/52.

2nd course: 0.5 ml., containing 2.5 mgm. antigen.

Exsanguinated on 22/4/52.

Rabbit 51

Antigen: human serum protein (10 mgm./ml.) iv. on alternate days.

1 course: dose 1	0.25 ml. (30/5/52)
doses 2 & 3	0.5 ml.
doses 4-7	1.0 ml.
dose 8	1.5 ml.
doses 9 & 10	2.0 ml.
doses 11-14	3.0 ml.
doses 15 & 16	5.0 ml. (5/6/52)
	<u>32.75 ml., containing 327.5 mgm. antigen.</u>

Exsanguinated on 15/7/52.

Rabbit 52

Antigen: human glomerulus suspension ip.

1 course: 2 ml. injected on 30/5/52, 31/5/52

1 ml. injected on 6 & 7/6/52, 13 & 14/6/52, 21 & 23/6/52.

Dose of glomeruli 1.25 gm.

Exsanguinated on 5/7/52.

Rabbits 53 and 54

Antigen: human tendon sections implanted ip. under ether or nembutal.

Dates and amounts implanted: 30/5/52 (0.5 gm.), 13/6/52 (0.5 gm.)
15/7/52 (0.5 gm.), 22/8/52 (1gm.)

Bled on 13/6/52, 15/7/52, 10/9/52.

Rabbits 66 and 67

Antigen: bovine chondroitin sulphate-vaccine iv. on alternate days.

1st course: doses 1 & 2	0.1 ml. (23/10/52)
doses 3 & 4	0.2 ml.
dose 5	0.4 ml.
dose 6	0.5 ml.
doses 7 & 8	0.75 ml.
dose 9	1.0 ml. (13/11/52)
	<u>4.0 ml., prepared from 4.0 ml.</u>
	<u>chondroitin sulphate.</u>

Bled on 25/11/52.

2nd course: dose 1	0.1 ml. (13/1/53)
dose 2	0.2 ml.
doses 3 - 5	0.5 ml.
doses 6 & 7	0.75 ml.
doses 8 & 9	1.0 ml.
doses 10 & 11	1.5 ml. (6/2/53)
	<u>8.3 ml., prepared from 3.0 ml.</u>
	<u>chondroitin sulphate.</u>

Bled on 18/2/53.

Rabbits 72 and 73

Antigen: human chondroitin sulphate - vaccine iv. on alternate days.

1st course: doses 1 - 9 as for rabbits 66 & 67 (1st course)
 doses 10 - 12 1.0 ml. (4/12/52)
 7.0 ml., prepared from 5.0 ml.
 chondroitin sulphate.

Bled on 16/12/52.

2nd course: as for rabbits 66 & 67 (2nd course) (13/1/53 - 6/2/53).

Bled on 18/2/53.

Rabbits 74 and 75

These animals received the 2nd course given to rabbits 72 & 73.

Bled on 18/3/53

Rabbits 78 and 79

Antigen: human glomerulus digest (No. 4) iv. on alternate days.

1 course: dose 1 0.1 ml. (3/3/53)
 dose 2 0.25 ml.
 doses 3 & 4 0.5 ml.
 dose 5 0.8 ml.
 dose 6 0.9 ml. (14/3/53)
 3.05 ml.

Bled on 26/3/53.

Rabbits 80 and 81

Antigen: Human glomerulus digest (no.4) - vaccine iv. on alternate days.

1 course: dose 1 0.1 ml. (3/3/53)
 dose 2 0.2 ml.
 doses 3 & 4 0.5 ml.
 doses 5 & 6 0.75 ml.
 doses 7 & 8 1.0 ml.
 dose 9 1.2 ml.
 dose 10 1.75 ml (24/3/53)
 7.75 ml., prepared from 4.0 ml.
 digest.

Bled on 5/4/53.

Rabbit 82

Antigen: human glomerulus suspension ip.

1st course: 1 ml. injected on 15 & 16/4/53
 1.5 ml. injected on 23 & 24/4/53, 30/4/53, 1/5/53,
 14 & 15/5/53
 1 ml. injected on 21/5/53. Dose of glomeruli 3.0 gm.

Bled on 3 & 12/6/53.

2nd course: 1 ml. injected on 30/6/53
 1.5 ml. injected on 1/7/53, 8 & 9/7/53, 15/7/53
 Dose of glomeruli 1.4 gm.

Exsanguinated on 10/8/53.

Rabbits 89 and 90

Antigen: human kidney suspension ip.

1 course: 1 ml. injected on 29/5/53, 4 & 6/6/53
 1.5 ml. injected on 11 & 12/6/53
 2 ml. injected on 20 & 22/6/53.
 Dose of kidney 2 gm.

Bled on 7 & 9/7/53.

Rabbits/

Rabbits 91, 93 and 95

Antigen: human glomerulus digest (No. 7)-vaccine iv. on alternate days.

1 course: dose 1	0.1 ml. (22/7/53)
dose 2	0.2 ml.
doses 3 - 5	0.4 ml.
doses 6 & 7	0.75 ml.
doses 8 - 10	1.0 ml.
dose 11	<u>1.5</u> ml. (9/8/53)
	7.5 ml., prepared from 3.5 ml. digest.

Thus, each rabbit received vaccine made from 1.16 ml. digest.

Bled on 21/8/53.

Rabbits 92, 94 and 96 (litter-mates of 91, 93 and 95)

Antigen: human glomerulus digest (No. 7) iv. on alternate days.

1 course; doses as for rabbits 91, 93 and 95

Dose of digest/rabbit 7.5 ml.

Bled on 21 & 23/8/53.

Rabbits 97 and 98

Antigen: vaccine only iv. on alternate days.

1 course: doses as for rabbits 91, 93 and 95

Rabbit 97 found dead on 17/8/53. Autopsy showed extensive coccidiosis, abscesses in kidneys and pleural effusions.

Contaminants isolated from kidney, but no streptococci.

Rabbit 98 bled on 21/8/53.

Rabbits 99 - 101

Antigen: human glomerulus digest (No.7) iv. on alternate days.

1 course: dose 1	0.2 ml. (7/8/53)
doses 2 & 3	<u>0.5</u> ml. (11/8/53)
	1.2 ml.

Bled on 21/8/53.

Rabbits 102 and 103

Antigen: human chondroitin sulphate (10 mgm./ml.) iv. on alternate days.

1 course: dose 1	0.1 ml. (24/10/53)
dose 2	0.2 ml.
doses 3 - 5	0.5 ml.
doses 6 & 7	0.75 ml.
doses 8 - 10	1.0 ml.
dose 11	<u>1.2</u> ml. (18/11/53)
	7.5 ml., containing 75 mgm. chondroitin sulphate.

Bled on 30/11/53.

Rabbit 122

Antigen: human ACTH, alum-precipitated

Operations: left adrenalectomy on 11/5/54; right adrenalectomy on 25/5/54.

1st course: dose 1	0.6 mgm in 1 ml. saline (3/6/54).
doses 2 - 4	1.2 mgm.in 2 ml. saline (5,21, 22/6/54).
dose 5	<u>1.8</u> mgm.in 3 ml. saline (23/6/54)
	6.0 mgm.

Half of each dose ip. and half subcutaneously (sc.).

Bled on 28/6/54.

2nd/

2nd course: dose 1 1.8 mgm. in 3 ml. saline (28/6/54)
 dose 2 3.6 mgm. in 6 ml. saline (29/6/54)
 5.4 mgm.

Routes of administration as above.

Exsanguinated on 6/7/54.

Rabbit 124

Antigen: human ACTH, alum-precipitated.

Operations: left adrenalectomy on 24/5/54; right adrenalectomy on 2/6/54.

1st course: as for rabbit 122 (1st course) (10/6/54 - 30/6/54)
 Bled on 5/7/54.

2nd course: as for rabbit 122 (2nd course) (5 & 7/7/54)

Total dose of ACTH 11.4 mgm.

Bled on 14 & 16/7/54.

Rabbits 127 and 128

Antigen: human post-menopausal gonadotrophin (HMG-20A)

Operations: castration on 25/5/54.

1st course: doses and routes of administration as for rabbit 122
 (3/6/54 - 27/6/54).

Bled on 28/6/54.

2nd course: doses and routes of administration as for rabbit 122
 (29 & 30/6/54).

Bled on 6/7/54.

3rd course: 1.8 mgm. in 3 ml. saline sc. on 26/9/54.

Bled on 15/9/54.

4th course: 1.8 mgm in 3 ml. saline sc. on 7/10/54.

Total dose of HMG-20A 15 mgm.

Exsanguinated on 26/10/54.

Rabbits 135 - 138

Antigen: human ACTH

Operations: left adrenalectomy on 19/10/54 (135 & 136) or
 21/10/54 (137 & 138); right adrenalectomy on
 2/11/54 (135 & 136) or 4/11/54 (137 & 138).

1st course: 2.5 mgm. alum-pptd. ACTH in 5 ml. saline intramuscularly (im.) on 18 & 19/11/54.

Bled on 22/12/54.

2nd course: 2.5 mgm. alum-pptd. ACTH in 5 ml. saline im. in two sites on 23/12/54.

Bled on 13/1/55.

3rd course: 2.5 mgm. ACTH in saline im. on 1/2/55

1.0 mgm. ACTH in saline iv. on 2/2/55.

Total dose of ACTH 11 mgm.

Bled on 5 & 10/2/55.

The doses and routes of administration used for these and later animals injected with hormone preparations, except for rabbits G3 and G4, were recommended by Mr. H. Proom, Wellcome Research Laboratories (1955).

Rabbit 141

Antigen: human thyrotrophic hormone (urinary extract).

Operation: bilateral thyroidectomy on 8/2/55

1st - 4th courses: 7.5 mgm. alum-pptd. TSH in 3.25 ml. saline
 im. in two sites on 15/3/55, 14/4/55, 5/10/55,
 7/11/55

Bled on 13/4/55, 9/5/55, 4/11/55, 30/11/55

5th course: 8 mgm. alum-pptd TSH in 4 ml. saline im. in two sites
 on 7/12/55

2.5 mgm. TSH in 0.5 ml. saline

iv. on 2 & 4/2/56.

Total dose of TSH 43 mgm.

Bled on 7/2/56

Rabbits 142 and 143

Antigen: human chondroitin sulphate (10mgm./ml.) iv. on alternate days.

1 course: as for rabbits 102 and 103 (11/2/55 - 4/3/55).

Dose of chondroitin sulphate 75 mgm.

Bled on 16/3/55.

Rabbits 144 and 145

Antigen: monkey kidney suspension ip.

1 course: 1 ml. injected on 1 & 2/3/55, 7 & 10/3/55, 15 & 16/3/55, 22 & 23/3/55.

Dose of kidney 1 gm.

Bled on 7/4/55.

Rabbits 146 and 147

Antigen: ox growth hormone.

1st course: 5 mgm. alum-pptd. hormone in 5 ml. saline im. in two sites on 15/3/55

Bled on 13/4/55

2nd course: 4 mgm. alum-pptd. hormone in 4 ml. saline im. on 14/4/55

Bled on 7/5/55

3rd course (rabbit 147 only): 2.5 mgm. alum-pptd. hormone in 2.5 ml. saline im. on 16/5/55
1 mgm. hormone in saline iv. on 20/5/55

Total dose of hormone 12.5 mgm.

Rabbit 147 bled on 21/5/55.

Rabbits 150 - 153

Antigen: acid-soluble rat collagen (0.1 per cent in 1/10,000 acetic acid ip.

1st course: 4 ml. injected on 20-23/7/55, 26-29/7/55, 2-5/8/55, 9-12/8/55, 15-19/8/55, 22-26/8/55, 30/8-2/9/55, 7 & 8/9/55

Dose of collagen 128 mgm.

Bled on 5/10/55

2nd course: 4 ml. injected on 8/10/55, 11-14/10/55, 19 & 20/10/55
Dose of collagen 28 mgm.

Bled on 3/11/55

3rd course: 4 ml. injected on 15-18/11/55, 22-25/11/55
Dose of collagen 28 mgm.

4th course: 4 ml. injected on 29/2/56, 1-3/3/56, 7-10/3/56
Dose of collagen 28 mgm.

Bled on 26/3/56.

Rabbits 156 and 157

Antigen: poliovaccine culture fluid iv. on alternate days

1 course: dose 1 - 2	0.5 ml. (2/8/55)
doses 3 - 4	1.0ml.
doses 5 - 6	1.5 ml.
doses 7 - 8	2.0 ml.
doses 9 - 10	3.0 ml.
dose 11	4.0 ml.
dose 12	5.0 ml. (26/8/55)
	25.0 ml.

Bled on 13/9/55.

Rabbit 158

Antigen: human thyrotrophic hormone (urinary extract)

Operation: bilateral thyroidectomy on 20/9/55

Injections and bleedings as for rabbit 141 from 5/10/55

Total dose of TSH 28 mgm.

Rabbits 160 - 164

Antigen: acid-soluble rat collagen (as for rabbits 150 - 153)

1st course: 4 ml. injected on 15 - 18/11/55, 22 - 25/11/55,
29/11 - 2/12/55

2nd course: 4 ml. injected on 1 - 4/2/56, 6 - 9/2/56, 13 - 14/2/56,
20 - 22/2/56, 1 - 3/3/56, 7 - 11/3/56

Total dose of collagen 140mgm.

Bled on 26/3/56.

Rabbits 169 and 170

Antigen: human kidney digest (No. 9) - adjuvants

1st course: 1 ml. digest-adjuvant mixture sc. in four sites on
2/2/56, 10/2/56, 18/2/56

Total dose of digest 1.26 ml.

Bled on 26/3/56

2nd course: 1 ml. digest in saline sc. on 30/3/56

1 ml. digest in saline iv. on 31/3/56

Bled on 14/4/56.

Antigen: human glomerulus suspension ip.

1st course: 1 ml. injected on 12 - 15/2/57, 14 - 16/3/57,
20 - 23/3/57, 26 - 28/3/57, 2 - 5/4/57

Dose of glomeruli 3.4 gm.

Bled on 22/4/57 and 4/6/57

2nd course: 1 ml. injected on 18 - 20/7/57

Dose of glomeruli 60 mgm.

Bled on 5 & 9/8/57.

Rabbits G3 and G4

Antigen: human growth hormone.

1st course: 1 ml. hormone-adjuvant mixture sc on 17/7/56,
24/7/56, 2/8/56

Bled on 10/9/56

2nd course: 5 mgm. alum-pptd. hormone in 2.5 ml. saline im. in
two sites on 11/9/56, 18/9/56

Total dose of hormone 16.9 mgm.

Bled on 2 & 4/10/56

Rabbit G7

Antigen: human thyrotrophic hormone (pituitary extract)

Operation: subtotal thyroidectomy on 16/10/56

1st course: 7.5 mgm. alum-pptd. TSH in 3.25 ml. saline im. in
two sites on 30/10/56

6.5 mgm. alum-pptd. TSH in 3 ml. saline im. in two
sites on 17/12/56

Bled on 10/1/57

2nd course: 4 mgm. alum-pptd. TSH in 2 ml. saline im. on 14/1/57

Bled on 4/2/57

3rd course: 2.5 mgm. TSH in 1 ml. saline iv. on 11/2/57

Bled on 14/2/57.

Rabbit G23

Antigen: human gamma globulin

1st course: 1 ml. protein-adjuvant mixture sc. in two sites on
3/6/57

2 ml. protein-adjuvant mixture sc, in four sites on
10/6/57, 17/6/57

Bled/

Bled on 22, 23 & 25/7/57

2nd course: 1 ml. gamma globulin in saline sc. on 26/7/57

0.1 ml. gamma globulin in saline ip. on 27/6/57

Bled on 30/7/57, 1, 2 & 7/8/57

The immune serum reacted with 1/10 human serum to a titre of 1/64 and with 1/1000 gamma globulin to a titre of 1/4.

Figs. 44 and 45 (p. 108a) show the reactions of this serum in Ouchterlony plates.

Guinea-pigs 14 - 19

Antigen: poliovaccine culture fluid (active anaphylaxis expt.)

Doses: 2.0 ml. fluid ip. on 18/7/55

10 ml. fluid iv. on 28/7/55

Result: no reaction.

Guinea-pigs 20 - 25

Antigen: poliovaccine culture fluid (passive cutaneous anaphylaxis expt.)

Doses: 0.1 ml. serum from rabbits 156 and 157 intradermally in separate sites on 14/11/55

0.1 ml. culture fluid and 0.25 ml. trypan blue (1 per cent) iv. six hours later.

Result: No reaction.

ANALYSIS OF IMMUNE SERA AND ELUATES

Antigens

Certain preparations, in addition to those used for immunisation were used in these tests.

The supernatant fluid of the kidney suspensions (see p. 186) was used in precipitin tests in the basement membrane experiments. It was prepared by spinning the suspensions for 15 min. at 5000 r.p.m. and removing a portion of the clear fluid.

The saline extract of kidney used in Section III was prepared by homogenising human kidney in the Waring Blender, preparing a 2 per cent suspension and spinning for 1 hr. at 14,000 r.p.m. in a refrigerated centrifuge (Rothenberg et al., 1956).

Rat, human or monkey red cells were prepared by collecting blood in 3.8 per cent sodium citrate, washing the red cells repeatedly in saline and making up a 5 per cent suspension in saline.

The Lancefield extract was prepared from the streptococci by following exactly the method of Mackie and MacCartney (1945).

Trypsinised glomeruli were prepared by a modification of the method of Milazzo (1957). Glomeruli were isolated as described on p. 186. A thick suspension was incubated for 2 hrs. at 37°C with 0.25 per cent Difco trypsin at pH 8 (borate buffer). The preparation was then spun and washed twice in saline. The sediment was resuspended in saline and homogenised at top speed in a Melco homogeniser for 2 hrs. The vessel containing the glomeruli was immersed in an ice bath during this procedure. The preparation was then spun for 20 min. at 3000 r.p.m. and the supernatant fluid separated and diluted with saline to match the opacity of tube 1 in the Brown series.

Trypsinised arterial reticulin was prepared from superior mesenteric/

mesenteric vessels, obtained at autopsy as shown on p. 204. The vessels were dissected free from the surrounding tissue and the intima and media obtained by drawing the vessels under a scalpel blade while exerting firm downward pressure on the blade. The intima and media were cut into small pieces and processed as for the glomeruli.

Blood vessel extracts were also prepared from superior mesenteric arteries, obtained as shown on p. 204. The vessels were freed from the surrounding tissue and cut into small pieces. The saline extract was prepared by incubating for 1 hr. at 37°C and spinning for 1 hr. at 14,000 r.p.m. in a refrigerated centrifuge. The supernatant fluid constituted the saline extract. The residue was treated with 5 M KCl for 15 min. at 37°C, spun and the supernatant fluid dialysed against running tap water overnight. The dialysed solution was spun for 1 hr. at 14,000 r.p.m. and used undiluted. The remaining solid material was incubated for 2½ hr. at 37°C with 500 mgm. Armour trypsin at pH 8 (borate buffer). It was then heated for 30 min. at 60°C, spun for 1 hr. at 14,000 r.p.m. and used undiluted.

For absorption of immune sera, wet organ preparations were packed solid and used in the proportion of 1 part to 3 parts of serum. Red cells were used in a concentration of 20 per cent in the serum. Normal serum was used in the proportion of 1 part to 9 parts of immune serum. Solid, dry preparations were used in the proportion of 1 mgm./ml. of immune serum.

Elution Methods

Glomeruli were isolated as described on p. 186 and stored in the deep freeze until used. Each preparation was then suspended in a suitable volume of saline and divided into six equal portions, five of which were used in the experiments and the sixth kept in reserve. The quantities of glomeruli in the portions varied from 0.1 to 0.5 gm. depending on the total quantity available. The portions were stored in the deep freeze until used.

Elution by heat (Korngold and Pressman, 1953). The glomeruli were spun down and resuspended in 5 ml. borate buffer at pH 8. They were then stood at 60°C for 15 min., the tubes spun for 10 min. at 3000 r.p.m. and the supernatant fluids separated.

Elution by saline and barium salts (Heidelberger and Kendall, 1936). The glomeruli were spun down and resuspended in 5 ml. 15 per cent saline. After incubation at 37°C for 1 hr., they were spun at 3000 r.p.m. for 10 min. and the supernatant fluids separated. The process was repeated and the supernatant fluids combined and dialysed for 48 hrs. against saline.

The residues were then washed once in normal saline, spun down and resuspended in 5 ml. saline. Saturated Ba(OH)₂ was added to bring the pH to 11 (0.25 - 1 ml. was sufficient) and the tubes stood for 1 hr. in an ice-bath. 1 ml. 10 per cent BaCl₂ was then added and the tubes replaced in the ice-bath for 15 min. They were then neutralised with 20 per cent acetic acid, spun for 10 min. at 3000 r.p.m., the supernatant fluids separated and dialysed against saline until the dialysate was free of Ba⁺⁺.

The/

The saline and barium salt eluates were then combined.

Elution by sodium hydroxide (Pressman and Sherman, 1951). The glomeruli were spun down and resuspended in 5 ml. saline. N-NaOH was added to bring the pH to 11, the tubes shaken and immediately spun for 10 min. at 3000 r.p.m. The supernatant fluids were then separated, brought to pH 7 with phosphate buffer, stood for 1 hr. at room temperature, spun and the precipitates discarded.

Elution by citric acid (Talmage et al., 1954). The glomeruli were spun down and resuspended in 5 ml. saline. 0.2 M citric acid was added to bring the pH to 3.2 and the tubes stood for 15 min. at room temperature. They were then spun for 10 min. at 3000 r.p.m. and the supernatant fluids neutralised with 0.5N-NaOH.

Control The glomeruli were spun down and resuspended in 5 ml. saline. They were incubated for 1 hr. at 37 C, spun for 10 min. at 3000 r.p.m. and the supernatant fluids separated.

Globulins from all the eluates were precipitated by adding $\frac{1}{2}$ vol. saturated $(\text{NH}_4)_2\text{SO}_4$ and standing overnight at 4 C. The tubes were then spun, the precipitates dissolved in 1 - 2 ml. saline and dialysed for 48 hrs. against saline. The volumes were adjusted to 2 ml. by addition of saline or dialysis against 15 per cent polyvinylpyrrolidone in phosphate buffer.

Serological Methods

Precipitin tests were performed by the interfacial ring and capillary tube methods. In the former, serum was pipetted into the tube and an equal quantity of antigen layered on top. The tests were read after incubation for 1 hr. at room temperature. In the latter, serum was drawn up into the tube, followed by an equal quantity of antigen, care being taken to avoid air bubbles. The tube was stood upright in plasticine, incubated for 2 hrs. at 37C and then stood overnight at 4 C.

Agglutination tests with trypsinised glomeruli and arterial reticulin were performed by the capillary tube method.

Agar-gel-diffusion tests were performed by a modification of the Ouchterlony technique. After several unsatisfactory trials with moulds, it was found that satisfactory results could be obtained when the cups were cut by hand with cork borers. A similar method has been used by Gell (1955). The medium was 2 per cent Difco agar, containing 0.85 per cent saline and 1/10,000 thiomersalate: it was poured in a single layer. Duplicate plates were poured for each new antigen-antibody system, one being incubated at 37 C, the other at room temperature. A moistened filter paper was placed inside the cover of the Petri dish during incubation and the cups refilled with saline when required. The plates were photographed with transmitted light from a Pointolite source.

Complement fixation tests were performed with 5 per cent sheep cells, 2 MHD of complement, a fixed concentration of antigen and serial dilutions of serum. Rabbit sera were incubated at 60 C, human at 56 C to inactivate complement. Serum, antigen, complement and cell controls were used in all tests. Preserved complement was used in the tests with animal sera, fresh complement with human sera. The complement titre was determined before each series of tests. The actual test was/

was performed in the following manner: 0.1 ml. quantities of serum, antigen and complement, plus 0.2 ml. saline were incubated for 30 min. at 37°C; 0.2ml. haemolytic system and 0.3 ml. saline were added and the tubes incubated for a further 30 min. at 37°C.

CONJUGATION OF SERA

The labelled antisera used in the basement membrane experiments were prepared by Mrs. Audrey Crossland, using fluorescein amine donated by Dr. A.H. Coons. The anti-collagen, -chondroitin sulphate, -tryptic digests, human and some anti-hormone sera were labelled by Dr. Lynda Roy, using fluorescein prepared by herself or Boots Pure Drug Co. The remaining anti-hormone sera, anti-human globulin and later anti-human kidney and -glomerulus sera were labelled by the author, using amine prepared by Boots. An attempt was made by the author to synthesise the amine, but was abandoned when arrangements were made to have the material prepared on a large scale by Boots.

Preparation of Fluorescein Isocyanate

The procedure used by Coons and by Mrs. Crossland and Dr. Roy is to bubble phosgene gas through a solution of the amine in acetone and to remove the gas at the end of the reaction by evaporating the acetone. The author has used phosgene-in-toluene (B.D.H.) instead of the gas and this makes the evaporation more difficult. The procedure has been as follows.

The requisite quantity of fluorescein, weighed as the amine, was dissolved in 3 - 4 ml. acetone, dried by storage over CaCl_2 . This was added drop-wise to 6 - 8 ml. phosgene-in-toluene at 45°C. The mixture was stood for 30 min., with frequent shaking, by which time all the precipitate had redissolved and the solution was a pale yellow-green. It was evaporated to dryness, using a waterpump to draw dried air through the solution. The phosgene was removed from the evaporate in a trap containing 20 per cent NaOH. The evaporation was accelerated by intermittently creating a vacuum in the apparatus. The dried isocyanate was immediately dissolved in the requisite quantity of acetone/dioxane and added dropwise to the reaction mixture.

Conjugation

The reaction mixture contained serum (or globulin, prepared by salting out with half-saturated ammonium sulphate), saline, carbonate-bicarbonate buffer, acetone and dioxane in the proportions recommended by Coons and Kaplan (1950). It was cooled in an ice-bath. After adding the isocyanate, the flasks containing the mixture were shaken for 18 hours at 4°C with a Microid flask shaker. The mixture was then dialysed against saline at pH 7 (phosphate buffer) for 48 hrs. and the globulin precipitated with half-saturated ammonium sulphate. The precipitate was spun down, dissolved in phosphate-buffered saline and dialysed against this solution until the dialysate was free from fluorescein when examined in ultra-violet light.

Acetone-dried Tissue Powders

Human muscle, sheep muscle, sheep liver and guinea-pig liver were used at various times in the preparation of these powders. 50 - 100 gm. of tissue in an equal volume of saline was homogenised/

homogenised in the Waring Blender and transferred to a large beaker. 4 vol. acetone were added, with constant stirring, and the solid matter allowed to settle. The supernatant fluid was removed by suction and the solid matter washed repeatedly with saline, until free of haemoglobin. 1 vol. saline and 4 vols acetone were then added, the contents spun and the supernatant fluid removed; the process was repeated once. The tissue was then suspended in acetone and transferred to a Buchner funnel, where it was dried for 2 - 4 hrs. The semi-dry tissue was further dried overnight at 37°C. The dried powder was stored in a dessicator and used in the proportion of 100 mgm./ml. of labelled antisera.

PREPARATION OF TISSUES

Two methods were used -- fresh-freezing and freeze-drying. In the former, blocks of tissue not more than 2 mm. thick were placed against the side of a test-tube, which was then plunged into a drikold-alcohol mixture at -78°C for 10 sec.. The tube was transferred to the deep-freeze for storage. Sections were cut in a freezing cabinet of the type described by Coons and Kaplan (constructed by the Pressed Steel Co., Oxford), using a Cambridge flat-cutting microtome, with the special "window" attachment to the knife-blade described by Coons et al. (1951). Sections were cut at 6 μ and thawed on to slides by warming with the tip of a finger. Gelatinised slides were used in the early work but were not used later, except in the indirect method. The sections were dried in a current of cold air. If not used immediately, they were stored at 4°C for up to 48 hrs.: longer storage leads to marked increase in auto-fluorescence. In order to obtain satisfactory sections of fresh-frozen lung, a block 2 mm. thick was fixed in absolute ethanol for 2 hrs., washed in phosphate buffer at pH 7 for 1 hr. and placed in 10 per cent gelatin overnight at 37°C. Next day the block was hardened, trimmed and frozen in the usual way. Sections were cut at 12 μ and washed in buffer for 5 min. before staining.

Freeze-drying was performed by an Altmann-Gersh method. In early experiments the tissue was quenched in dichloro-difluoromethane (Arcton 6, I.C.I.) cooled to -150°C to -160°C in liquid air in a Dewar flask (Bell, 1952). The dehydration apparatus used was the freeze-drying unit No. 2 described by Glick and Malmstrom (1952), adapted to fit directly on to an Edwards Speedivac 2S/20 vacuum pump. The tube was surrounded by a drikold-diethylene glycol mixture in a Dewar flask and the water trap filled with liquid oxygen by day and drikold overnight. Satisfactory dehydration was obtained after 48 hrs. The tissue was transferred to paraffin wax in a vacuum embedding oven for 10 min. and then embedded. Blocks were stored in a dessicator. Some trouble was experienced in flattening the sections on to slides, but this was overcome by floating them on methanol after cutting. Before treatment with labelled antisera, the sections were dewaxed in xylol, fixed in methanol for 30 min. at 37°C and washed in phosphate buffer at pH 7. In later experiments quenching was performed in isopentane cooled to -150°C to -160°C in liquid nitrogen and dehydration and embedding in an Edwards Tissue Drying Apparatus.

TREATMENT OF SECTIONS WITH LABELLED ANTISERA

The/

The method of Coons and Kaplan was followed in early experiments, namely, treatment for 2 - 3 hrs. at room temperature, followed by washing in two changes of phosphate buffer for 10 min. and mounting in reagent glycerol containing 10 per cent of the same buffer. Later on, variations in the staining time were introduced, such as 30 min. at 37°C, overnight at 4°C and, finally, 30 min. at room temperature, all of which gave equally satisfactory results.

Inhibition tests were performed by using unlabelled sera for $\frac{1}{2}$ - 3 hrs., washing in buffer and treating with labelled sera for $\frac{1}{2}$ - 1 $\frac{1}{2}$ hrs.

In the indirect method, unlabelled immune serum and labelled anti-globulin serum were each used for 30 min. at room temperature.

EXAMINATION OF SECTIONS

Two sets of apparatus were used. First, a 250-watt, high-pressure mercury vapour lamp (Mazda ME/D 26) with a Watson monocular microscope. A Corning 5840 ultra-violet filter and a 1 inch cuvette of 10 per cent copper sulphate were used to filter the light and a quartz or glass collecting condenser and a stainless steel reflecting mirror. Equally satisfactory results were obtained with either type of condenser. A Wratten 2B filter and a glass cell containing 2 per cent sodium nitrite (Metcalf and Patton, 1944) were used in the eyepiece. Second, a Leitz BX20 fluorescence microscope, using a carbon arc or a 150-watt compact source mercury vapour lamp (Philips ME/D 103803).

Photographs were taken with the first apparatus on Ilford Special Rapid Panchromatic plates, using exposure times of 20 - 60 min. and Ilford PanF 35 mm. film, using exposure times of 25 sec. - 4 min. No photographs have yet been taken with the second apparatus.

SOURCE OF TISSUE USED FOR IMMUNISATION, ETC.

Ref. No.	Hospital	Sex	Age	Diagnosis	Tissue	Disposal
EHA 1581	B	F	8	multiple injuries	kidney	immunisation of rabbit 48
EHA 1660	B	M	66	emphysema: cardiac failure	glomeruli	immunisation of rabbit 52
PM322/52	RIE	M	65	multiple injuries		
PM238/52	RIE	F	32	bronchiectasis: cardiac failure	kidney, liver,	staining with labelled
PM282/52	RIE	F	50	rheumatic heart disease	spleen, thyroid,	antisera
PM325/52	RIE	M	66	cup arthroplasty: pulm. embolism	etc.	tendon from PM325 for immunisation of rabbits 53 & 54
PM516/52	RIE	F	21	congenital heart disease		
PM510/52	RIE	M	50	carcinoma of bladder	costal cartilages	chondroitin sulphate for immunisation of rabbits 72-75, 102, 103, 142, 143
PM700/52	RIE	M	58	volvulus	glomeruli	tryptic digest No. 4
PM722/52	RIE	M	79	multiple injuries		
PM731/52	RIE	M	34	Hodgkin's disease	glomeruli	immunisation of rabbit 82 (1st course)
PM 94/53	RIE	F	34	multiple myeloma		
PM 95/53	RIE	M	47	ependymoma		
PM123/53	RIE	F	46	subarachnoid haemorrhage		
PM164/53	RIE	M	62	glioblastoma multiforme		
EHA 1941	B	F	36	carcinoma of colon		
PM 48/53	RIE	F	23	rheumatic heart disease	kidneys and	portions of whole kidney for immunisation of rabbits 89 & 90
PM312/53	RIE	F	79	coronary thrombosis	glomeruli	
PM386/53	RIE	M	65	cirrhosis of liver		
PM387/53	RIE	F	49	hypertensive cardiac failure		
PM392/53	RIE	M	66	bronchial carcinoma		portions of glomerular suspension for immunisation of rabbit 82 (2nd course)
PM396/53	RIE	M	12	congenital heart disease		
PM398/53	RIE	M	69	coronary thrombosis		
PM401/53	RIE	M	75	duodenal ulcer: haematemesis		
MHA 5211	EG	M	49	bronchial carcinoma		remainder of glomeruli for tryptic digest No. 7
MHA 5212	EG	M	69	bronchial carcinoma		
MHA 5229	WG	F	45	rheumatic heart disease		
MHA 5264	WG	F	60	hypertensive cardiac failure		
MHA 5265	NG	F	69	malignant astrocytoma		
EHA 1993	B	M	63	bronchial carcinoma		
MHA 5299	EG	F	54	bronchial carcinoma	kidneys	tryptic digest No. 8
PM 48/53	RIE	F	23	rheumatic heart disease		
MHA 5472	EG	F	60	carcinoma of oesophagus	kidneys	tryptic digest No. 9
MHA 5477	WG	F	25	rheumatic heart disease		
MHA 5480	EG	M	71	bronchial carcinoma		
PM769/55	RIE	M	33	head injury (blood-group O)	costal cartilages	chondroitin sulphate
				mesenteric arteries		blood vessel extracts
PM784/55	RIE	M	73	head injury (blood-group A)	costal cartilages	chondroitin sulphate
PM 32/56	RIE	M	13	congenital heart disease	mesenteric arteries	blood vessel extracts
PM 72/56	RIE	F	43	congestive cardiac failure		
PM104/57	GRI	M	58	rheumatic heart disease	glomeruli and	glomeruli used for immunisation of rabbits 169 & 170
PM440/57	GRI	M	49	duodenal ulcer: haematemesis	kidney	kidney for staining with nephritic sera
PM252/57	GRI	M	28	malignant hypertension	glomeruli	trypsinised glomeruli
				mesenteric arteries		arterial reticulin
				kidney		staining with nephritic sera

PM353/56	GRI	F	39	rheumatic heart disease	pituitary	staining with labelled
PM359/56	GRI	M	40	cerebral infarction	kidney, liver	antisera to pituitary
PM398/56	GRI	M	61	carcinoma of bladder	or adrenal	hormones
PM399/56	GRI	M	48	multiple myeloma		
PM405/56	GRI	M	64	congestive cardiac failure		
PM406/56	GRI	M	46	duodenal ulcer: haematemesis		
PM412/56	GRI	F	48	burns		
PM171/58	GRI	M	49	myocardial infarction	glomeruli	normal control in elution experiments

SOURCE OF SERA AND ELUATES

Serial Number	Hospital and Ward	Initials	Sex	Age	Dates of admission	Date(s) serum obtained	Diagnosis	Result
1	RIE/29	A.F.	M	33	28/2/53	9/3/53	chronic glomerulonephritis	I.S.Q.
2	RIE/29	A.F.	M	13	29/1/53	10/3/53	acute glomerulonephritis	recovered
3	RIE/26	B.H.	M	51	23/1/53	11/3/53	" "	"
7	RIE/26	H.O.	M	60	19/5/53	1/6/53	acute exacerbation of chronic glomerulonephritis	improved
8	RIE/29	T.M.	M	16	1/6/53	3/6/53	acute "	recovered
9	RIE/30	J.B.	F	64	8/6/53	10/6/53	chronic "	I.S.Q.
10	SCE/1	G.C.	M	4	22/6/53	25/6/53	acute "	recovered
11	SCE/2	C.L.	F	5	27/5/53	2/7/53	" "	"
12	SCE/2	M.P.	F	5	28/4/53	1/7/53	subacute "	improved
13	RIE/29	V.B.	M	31	27/4/53	20/7/53	acute " (recurrence)	"
14	RIE/29	J.S.	M	48	20/7/53	21/7/53	acute "	recovered
15	RIE/27	H.L.	F	17	17/3/50*	19/8/53	chronic "	died: PM511/53†
16	RIE/28	J.D.	F	23	8/5/53			died: PM706/53†
					16/7/53	13/10/53	type II nephritis	
17	SCE/1	G.K.	M	?	27/1/52*	21/10/53	anaphylactoid purpura with nephritis	recovered
18	RIE/23	J.H.	M	24	19/10/53	26/10/53	chronic glomerulonephritis	I.S.Q.
19	RIE/32	D.S.	M	43	23/9/53	28/10/53	type II nephritis	improved
20	SCE/	R.I.	M	5	14/9/53	15/9/53	acute glomerulonephritis	recovered
21	EG/	M.B.	F	29	21/11/53	23/11/53	" "	"
23	SCE/1	F.W.	F	10	3/12/53	8/12/53	" "	"
24	RIE/26	R.H.	M	33	28/2/53	9/3/53	type II nephritis	I.S.Q.
27	SCE/1	N.H.	F	6	1/4/54	9/4/54	acute glomerulonephritis	recovered
29	B/Q8	A.B.	M	44	28/4/54	28/4/54	" "	"
30	SCE/5	B.L.	M	4	8/5/54	11/5/54	" "	"
31	WG/CW	A.C.	M	5	12/5/54	14/5/54	" "	"
32	RIE/26	A.T.	M	57	25/4/54	24/5/54	type II nephritis	died: PM416/54
35	L/5	R.F.	M	13	18/2/54	19/5/54	polyarteritis nodosa	died: PM 1HA685
36	RIE/30	C.B.	F	23	25/11/54	10/1/55	disseminated lupus erythematosus	improved
38	SCE/5	J.H.	F	6	29/12/54	12/1/55	subacute glomerulonephritis	died: PM55/3
39	SCE/5	S.C.	M	4	5/1/55	28/1/55	anaphylactoid purpura	recovered
40	SCE/1	A.H.	F	8	1/11/54	9/2/55	(with nephritis)	improved
41	SCE/5	B.S.	M	4	9/2/55	10/2/55	acute glomerulonephritis	recovered
42	SCE/5	I.McP.	F	7	19/2/55	22/2/55	" "	"
43	SCE/1	J.P.	M	6	21/2/55	26/2/55	type II nephritis	died: PM55/25
44	SCE/5	P.B.	F	7	26/2/55	1/3/55	anaphylactoid purpura	recovered
46	SCE/1	H.A.	M	5	28/8/55	31/8/55	" " with nephritis	"
48	SCE/5	S.Y.	F	3	27/1/51	7/9/55	acute nephritis	"
					12/11/55	14/11/55	(recurrent)	"

49	SCE/1	G.S.	M	2	12/9/55	12/9/55	anaphylactoid purpura	recovered
50	SCE/5	D.D.	F	3	20/10/55	20/10/55	acute glomerulonephritis	"
52	SCE/5	C.G.	F	7	8/9/54	10/9/54	anaphylactoid purpura	improved
					9/11/55	10/11/55	with nephritis	
53	SCE/2	G.P.	M	2	3/1/56	4/1/56	anaphylactoid purpura	recovered
							with nephritis	
54	SCE/1	N.C.	F	6	23/1/56	25/1/56	anaphylactoid purpura	improved
							with nephritis	
55	SCE/1	V.C.	F	10	26/1/56	27/1/56	anaphylactoid purpura	recovered
						8/2/56	with nephritis	
56	L/CW	C.H.	F	7	7/2/56	13/2/56	anaphylactoid purpura	"
57	SCE/3	T.S.	M	3	15/2/56	16/2/56	"	"
59	L/CW	A.D.	M	9	22/2/56	29/2/56	"	"
							with nephritis	
60	SCE/5	E.M.	M	9	29/2/56	1/3/56	anaphylactoid purpura	"
							with nephritis	
62	R/5	J.M.	F	25	19/8/55	3/10/55 [†]	disseminated lupus	died: PM EHA2694
							erythematosus	
63	WG/C4	C.W.	F	15	1/6/54	10/11/54	disseminated lupus	died: PMA/408/95/55
							erythematosus	
64	SCE/2	F.S.	F	?	?	22/3/56	anaphylactoid purpura	recovered
65	SCE/5	M.T.	F	5	28/3/56	30/3/56	"	"
67	BID/9E	J.D.	M	6	13/6/56	14/6/56 [†]	acute glomerulonephritis	died: PM B128/56
68	H/2	A.W.	M	64	28/1/57	22/2/57 [†]	disseminated lupus	died: PM 36/57 [†]
							erythematosus	
69	NGH/1	A.C.	M	45	10/9/51		polyarteritis nodosa	died: PM MHA5837
					3/4/52	4/7/52		
					26/1/56			
70	RIE/22	T.B.	M	46	20/7/55	22/7/55 [†]	acute glomerulonephritis	died: PM 496/55
							with subacute bacterial	
							endocarditis	
71	WG/A6	J.B.	M	32	18/4/52	7/5/52 [†]	disseminated lupus	died: PM MHA4775
							erythematosus	
72	NG/1	M.B.	F	37	17/2/50	13/11/50 [†]	disseminated lupus	died: PM MHA4015
							erythematosus	
73	NG/1	M.McG.	F	30	24/11/51	25/11/51 [†]	disseminated lupus	died: PM MHA 4590
							erythematosus	
74	NG/1	G.M.	F	14	16/1/50	22/4/50 [†]	disseminated lupus	died: PM MHA3709
							erythematosus	
75	GRI/6	C.S.	F	15	28/5/57		subacute glomeruloneph-	improved
					23/7/57	1/8/57 [†]	ritis	
76	GRI/9	W.K.	M	33	19/10/57	27/10/57	chronic glomerulonephritis	died: PM 408/57
77	SCG/	C.McF.	M	2	14/7/56	5/7/56	type II nephritis	improved
						31/7/56		
						8/8/56		
						29/8/56		
80	GRI/37	W.McC.	M	37	?	12/12/57	chronic cholecystitis	recovered
81	GRI/38	E.K.	F	23	21/12/57	23/12/57	acute appendicitis	"
82	GRI/35	F.McM.	M	23	6/1/58	7/1/58	"	"
86	GRI/37	L.McA.	M	38	11/1/58	16/1/58	"	"
87	GRI/37	R.T.	M	58	11/1/58	16/1/58	"	"
88	GRI/33	M.O.M.	F	18	13/1/58	16/1/58	"	"
89	GRI/33	P.McG.	F	18	14/1/58	16/1/58	"	"
90	GRI/33	E.K.	F	37	15/1/58	16/1/58	"	"
91	GRI/33	C.U.	F	23	14/1/58	16/1/58	"	"
92	S/	R.P.	M	39	9/12/57	17/1/58 [†]	acute glomerulonephritis	died: PM St8/58 [†]
93	GRI/31	J.H.	M	14	14/1/58	18/1/58	acute appendicitis	recovered
94	GRI/37	W.C.	M	17	17/1/58	24/1/58	"	"
95	GRI/27	E.R.	F	65	2/1/58	24/1/58	chronic cholecystitis	"
96	GRI/36	M.S.	F	36	13/1/58	24/1/58	"	"

97	GRI/36	M.S.	F	23	20/1/58	24/1/58	acute appendicitis	recovered
98	GRI/36	I.W.	F	53	20/1/58	24/1/58	chronic cholecystitis	"
99	GRI/36	H.M.	F	39	20/1/58	24/1/58	acute appendicitis	"
100	GRI/39	M.G.	F	46	18/1/58	25/1/58	chronic cholecystitis	"
101	GRI/31	T.H.	M	15	18/2/58	20/2/58	acute appendicitis	"
102	GRI/32	T.M.	M	19	18/2/58	20/2/58	" "	"
103	GRI/32	J.H.	M	11	18/2/58	20/2/58	" "	"
105	GRI/32	J.R.	M	18	1/4/58	5/4/58	" "	"
108	GRI/32	P.G.	M	32	5/4/58	10/4/58	" "	"
109	GRI/32	J.C.	M	24	5/4/58	10/4/58	" "	"
110	SCG/	J.G.	F	1	23/6/57	-	acute glomerulonephritis	died: PM 9378†
111	GRI/9	T.E.	M	33	7/4/58	18/4/58†	" "	died: PM 166/58†
114	GRI/36	L.O'N.	F	39	14/4/58	19/4/58	acute appendicitis	recovered
115	S//4	A.M.	M	15	28/12/53	-	acute glomerulonephritis	died: PM 65/53†
116	RIE/26	A.D.	M	59	30/12/53	-	amyloidosis (primary)	died: PM 36/54†
117	DWF	J.W.	M	40	18/4/53	-	amyloidosis (ankylosing spondylitis)	died: PM EHA 1995†
119	GRI/33	A.J.	F	42	23/4/58	26/4/58	chronic cholecystitis	recovered

Key to abbreviations, etc.

B	Bangour Hospital
BID	Belvidere Hospital, London Road, Glasgow
DWF	Dunfermline and West Fife Hospital
EG	Eastern General Hospital, Edinburgh
GRI	Glasgow Royal Infirmary
H	Hairmyres Hospital, East Kilbride
L	Leith Hospital
NG	Northern General Hospital, Edinburgh
R	Roodlands Hospital, Haddington
RIE	Edinburgh Royal Infirmary
S	Strathclyde Infectious Diseases Hospital, Motherwell
SCE	Royal Hospital for Sick Children, Edinburgh
SCG	Royal Hospital for Sick Children, Glasgow
WG	Western General Hospital, Edinburgh
CW	Childrens' Wing
*	serum obtained on subsequent admission
†	glomeruli kept for elution
‡	serum obtained at autopsy
§	detailed abstract in thesis, Section III, p.138 - 139